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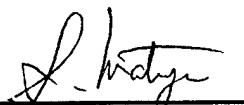
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## INTRODUCTION

Recently, it became evident that the escaping from the cell suicide program is one of the major reasons of the development of malignant cancer cells including breast tissue. The cell suicide program is called “apoptosis”. Bcl-2 family proteins are the major players in the regulation of apoptosis, and we explored the molecular mechanism of Bcl-2 family protein’s action through the innovative approach to study apoptosis which utilizes yeast genetics to explore the cell death mechanism of human cells. We discovered 1) Mitochondrial proton-pump function is required for Bax to induce apoptosis, 2) Bax-inhibitor-1 (BI-1) was discovered as a suppressor of Bax function to induce cell death. 3) BAR was discovered as a suppressor of Bax- and Fas-induced cell death, 4) Bcl-2 family proteins have the ion-channel formation activity in vitro and the protein sequences responsible for this activity is indispensable for their cell death regulating function, 5) Mitochondrial-induced cytosol acidification is a obligatory step for mitochondria-dependent cell death pathway. These findings provide the new strategies to develop anti-cancer drugs and the diagnostic-methods to detect malignant breast cancer cells. Followings are the summary of the each of our discovery.

## BODY

The followings are the summary of the achievement.

1) Mitochondrial proton-pump is required for Bax action to induce apoptosis.

The proapoptotic mammalian protein Bax associates with mitochondrial membranes and confers a lethal phenotype when expressed in yeast. By generating Bax-resistant mutant yeast and using classical complementation cloning methods, subunits of the mitochondrial F0F1-ATPase proton pump were determined to be critical for Bax-mediated killing in *S. cerevisiae*. A pharmacological inhibitor of the proton pump, oligomycin, also partially abrogated the cytotoxic actions of Bax in yeast. In mammalian cells, oligomycin also inhibited Bax-induced apoptosis and activation of cell death proteases. The findings imply that an intact F0F1-ATPase in the inner membrane of mitochondria is necessary for optimal function of Bax in both yeast and mammalian cells. (These findings were published In : Molecular Cell 1998 Feb;1(3):327-36 . “The Mitochondrial F0F1-ATPase proton pump is required for function of the proapoptotic protein Bax in yeast and mammalian cells.” by Matsuyama S, Xu Q, Velours J, Reed JC)

## 2) Discovery of Bax-inhibitor (BI-1) gene

The mammalian proapoptotic protein Bax confers a lethal phenotype when expressed in yeast. By exploiting this phenotype, we have identified a novel human Bax inhibitor, BI-1. BI-1 is an evolutionarily conserved integral membrane protein containing multiple membrane-spanning segments and is predominantly localized to intracellular membranes, similar to Bcl-2 family proteins. Moreover, BI-1 can interact with Bcl-2 and Bcl-XL but Bax or Bak, as demonstrated by in vivo cross-linking and coimmunoprecipitation studies. When overexpressed in mammalian cells, BI-1 suppressed apoptosis induced by Bax, etoposide, staurosporine, and growth factor deprivation, but not by Fas (CD95). Conversely, BI-1 antisense induced apoptosis. BI-1 thus represents a new type of regulator of cell death pathways controlled by Bcl-2 and Bax. (These findings were published in Molecular Cell 1998 Feb;1(3):337-46 . “Bax inhibitor-1, a mammalian apoptosis suppressor identified by functional screening in yeast.” Reported by Xu Q, Reed JC)

### 3) Discovery of BAR gene

A novel apoptosis regulator, BAR, was identified using a yeast-based screen for inhibitors of Bax-induced cell death. The BAR protein contains a SAM domain which is required for its interaction with Bcl-2 and Bcl-XL and for suppression of Bax-induced cell death both in mammalian cells and yeast. In addition, BAR contains a Death Effector Domain (DED) responsible for its interaction with DED-containing pro-caspases and suppression of Fas-induced apoptosis. Furthermore, BAR can bridge pro-caspase-8 and Bcl-2 into a protein complex. The BAR protein is anchored in intracellular membranes where Bcl-2 resides. Over-expression of BAR therefore may define a mechanism for simultaneously suppressing two major apoptosis pathways: Fas/death receptor and Bax/mitochondria.

(submitted to Proc. Natl. Acad. Sci. USA 1999)

#### 4) Ion-channel formation activity of Bcl-2 and Bax

We explored whether the putative channel-forming fifth and sixth alpha-helices of Bcl-2 and Bax account for Bcl-2-mediated cell survival and Bax-induced cell death in mammalian cells and in the yeast *Saccharomyces cerevisiae*. When alpha5-alpha6 were either deleted or swapped with each other, the Bcl-2Deltaalpha5alpha6 deletion mutant and Bcl-2-Bax(alpha5alpha6) chimeric protein failed to block apoptosis induced by either Bax or staurosporine in human cells and were unable to prevent Bax-induced cell death in yeast, implying that the alpha5-alpha6 region of Bcl-2 is essential for its cytoprotective function. Additional experiments indicated that, although alpha5-alpha6 is necessary, it is also insufficient for the anti-apoptotic activity of Bcl-2. In contrast, deletion or substitution of alpha5-alpha6 in Bax reduced but did not abrogate apoptosis induction in human cells, whereas it did completely nullify cytotoxic activity in yeast, implying that the pore-forming segments of Bax are critical for conferring a lethal phenotype in yeast but not necessarily in human cells. BaxDeltaalpha5alpha6 and Bax-Bcl-2(alpha5alpha6) also retained the ability to dimerize with Bcl-2. Bax therefore may have redundant mechanisms for inducing apoptosis in mammalian cells, based on its ability to form alpha5-alpha6-dependent channels in membranes and to dimerize with and

antagonize anti-apoptotic proteins such as Bcl-2. (These findings were published in Journal of Biological Chemistry 1998 Nov 20;273(47):30995-1001 .“Cytoprotection by Bcl-2 requires the pore-forming alpha5 and alpha6 helices.” by Matsuyama S, Schendel SL, Xie Z, Reed JC)

5) pH-regulation of mitochondria and cytosol for the induction of cell death.

Mitochondria trigger apoptosis by releasing caspase-activators, including cytochrome c (cytC). Using a pH-sensitive Green Fluorescence Protein, we show that mitochondria-dependent (Bax, Staurosporine, UV) but not - independent (Fas) apoptotic stimuli induce rapid, Bcl-2-inhibitable mitochondrial alkalinization and cytosol acidification, prior to cytC release, caspase activation, mitochondrial swelling and depolarization. Activation of cytosolic caspases by cytC is minimal at neutral but maximal at acidic pH, suggesting a role for mitochondria-induced cytosol acidification. Cytosol acidification, cytC release, and caspase activation were suppressed by oligomycin, a  $F_0F_1$ -ATPase/proton-pump inhibitor, but not by caspase inhibitors. Ectopic expression of Bax in wild-type but not  $F_0F_1$ /proton-pump-deficient yeast similarly resulted in mitochondrial matrix alkalinization, cytosol acidification, and cell death. These findings implicate the  $F_0F_1/H^+$ -pump in intracellular pH alterations which facilitate caspase activation during the mitochondria-dependent apoptotic program.

(These findings were submitted to Nature Cell Biology (1999)

## The list of publications

- (1) Matsuyama S, Xu Q, Velrous J, Reed JC (1998) F0F1-proton-pump ATPase is required for the function of a pro-apoptotic protein, Bax, both in yeast and mammalian cells. *Molecular Cell* 1: 327-336.
- (2) Xie Z, Schendel SL, Matsuyama S, Reed JC (1998) Acidic pH promotes dimerization of Bcl-2 family proteins. *Biochemistry* 37:6410-6418.
- (3) Matsuyama S (1998) Molecular mechanism of Bax-induced cell death. *Cell Technology* 17:891-898 (Japanese article).
- (4) Xu Q, Jurgensmeier JM, Zha H, Matsuyama S, Reed JC (1998) Exploiting yeast for the investigation of mammalian proteins that regulate programmed cell death. *Apoptosis Detection and Assay Methods*, Edited by Li Zhu and Jerald Chun, BioTechniques Books, Natick, MA. Page 93-115.
- (5) Reed JC, Jurgensmeir J, Matsuyama S (1998) Bcl-2 family proteins and mitochondria (Review). *Biochemica et Biophysica Acta*, 1366, 127-137.

(6) Marzo I, Brenner C, Zamzami N, Jurgensmeier JM, Susin SA, Vieira HLA, Prevost MC, Xie Z, Matsuyama S, Reed JC, Kroemer G. (1998) Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science*, 281. 2027-2031.

(7) Matsuyama S, Schendel SL, Xie Z, Reed JC (1998) Cytoprotection by Bcl-2 requires the pore-forming  $\alpha$ 5 and  $\alpha$ 6 helices. *Journal of Biological Chemistry* 273. 30995-31001.

(8) Matsuyama S (1999) Mitochondria dependent cell death pathway. In "Apoptosis and the digestive organ-diseases" (Edited by H. Ishi), p.p. 31-46 (Japanese article)

(9) Matsuyama S, Nouraini S, Reed JC (1999) Yeast as a tool for apoptosis research. *Current Opinion in Microbiology* 2:618-623.

(10) Xu Q, Matsuyama S, Reed JC (1999) Utilization of yeast genetics to explore mammalian cell death mechanism. *Methods in Enzymology* (in press)

(11) Matsuyama S (1999) Advantages of the utilization of yeast genetics to study apoptosis mechanism. *Experimental Medicine* (in press, Japanese article).

(12) Matsuyama S, Llipos J, Devraux Q, Tsien R, Reed JC (1999) Mitochondrial H<sup>+</sup> efflux: An early and obligatory step in apoptosis preceding cytochrome c release, caspase activation, and permeability transition. *Submitted to Nature Cell Biology*.

(13) Nuoraini S, Six E, Matsuyama S, Krajewski S, Reed JC (2000). The putative pore forming-domain of Bax regulates mitochondrial localization and interaction with Bcl-XL. *Mol Cell Biol (IN PRESS)*.

(14) Zhang H, Huang Q, Matsuyama S, Ke N, Godzik A, and Reed JC (2000). *Drosophila* Pro-Apoptotic Bcl-2/Bax Homologue Reveals Evolutionary Conservation of Cell Death Mechanisms. *Submitted to Nature cell Biology*

## Yeast as a tool for apoptosis research

Shigemi Matsuyama, Shahrzad Nouraini and John C Reed\*

Apoptosis is a unique cell suicide process that plays important roles in a wide variety of developmental and normal physiological processes in animal species, and causes diseases when inappropriately controlled. Although yeast do not possess the proteases ultimately responsible for the morphological events recognized as apoptosis, these simple unicellular eukaryotes can serve as a powerful tool for apoptosis researchers. Ectopic expression of several human and animal apoptosis proteins in either budding or fission yeast results in phenotypes that create opportunities for genetic screens. Recent exploitation of yeast as tools for studying human apoptosis-regulatory proteins has yielded novel insights into cell death mechanisms, suggesting strategies for identification of genes and drugs that modulate the functions of proteins involved in apoptosis control.

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### Abbreviations

BI-1	Bax inhibitor-1
IAP	inhibitor of apoptosis protein
PCD	programmed cell death
ts	temperature sensitive

### Introduction

Apoptosis is the term used to describe a type of cellular suicide that can occur in nearly all animal cells [1]. In adult humans, typically 50–70 billion cells are eradicated via this cell suicide mechanism on a daily basis, thus making room for the equivalent number of cells produced daily through cell division. In its strictest use, the term 'apoptosis' represents a morphological definition, referring to the characteristic changes in cell ultrastructure that are visible in part by light microscopy but appreciated in their fullest by transmission-electron microscopy. These morphological and ultrastructural changes include plasma membrane blebbing, cell rounding and shrinkage, chromatin condensation and nuclear fragmentation, culminating in the ultimate breakage of the cells into small membrane-surrounded fragments referred to as apoptotic bodies [1]. Within the context of multicellular animals, these apoptotic corps and their fragments are cleared by neighboring viable cells through phagocytosis, without inciting inflammatory responses. When apoptosis occurs as a normal developmental or physiological process, it is often referred to as programmed cell death (PCD). Exceptions exist, however, where normal developmental cell deaths do not encompass the full panoply of morphological changes typically recognized in classic PCD [2]. Thus, 'apoptosis' and 'PCD' are not necessarily synonymous terms.

In recent years, it has been determined that the biochemical event responsible for the phenomenon of apoptosis is the activation of a family of intracellular cysteine proteases (caspases) with specificity for cleavage of substrates at aspartyl residues [3]. The proteolytic cleavages mediated by caspases are responsible directly or indirectly for the various molecular and morphological features of apoptosis. However, because not all of the events associated with apoptosis result directly from protein cleavage by caspases, a subset of them may be produced or mimicked by alternative routes not involving these enzymes. For example, although no caspases have been identified to date in plants, elements of an apoptosis-like process in plants have been observed, particularly in the context of the 'hypersensitive' response to pathogens, where some cells sacrifice themselves to limit the replication and thus spread of microbes or viruses [4]. Though not qualifying as apoptosis on morphological grounds, PCD also plays a wide variety of important roles in plant development (reviewed in [5,6]). Moreover, in animal cells, a cell death commitment point has been mapped upstream of caspases, involving the participation of mitochondria as the engines of cell death and resulting in a caspase-independent cell suicide process which morphologically does not resemble apoptosis (reviewed by [7\*]). Thus, caspase-independent cell suicide mechanisms exist, which may be more ancient in an evolutionary sense than caspases and apoptosis. Indeed, because it could be disadvantageous for an animal to carry a lifetime's dead cells, it could be argued that caspases and the phenomenon of apoptosis evolved after the split of the plant and animal kingdoms as a means of very neatly disposing of corps in animals, where motility of the organisms is often an important aspect of daily function and survival. Yeast cells have no caspase homologs, yet these simple organisms can help us to dissect the complicated cell death pathways used in higher eukaryotic cells. In this review, we examine some of the reasons why cell suicide mechanisms might exist even in unicellular organisms and review current information about ways that yeast based assays can be used as tools for apoptosis research.

### Cell suicide responses in unicellular organisms

Cell suicide responses have been documented in unicellular organisms, including bacteria and some eukaryotic cells [2]. Why would it be useful for organisms to possess a mechanism for self-eradication? The answer seems likely to come from appreciating that unicellular organisms give rise to colonies or populations of cells, all possessing the same genome derived from a common ancestor. Thus, cellular behaviors that sacrifice some of the members of this population so that others can survive ultimately ensure propagation of the original genome to future generations. The theoretical evolutionary pressures for cell suicide among unicellular species include the following examples.

48. Li R: **Bifurcation of the mitotic checkpoint pathway in budding yeast**. *Proc Natl Acad Sci USA* 1999, **96**:4989-4994.

Bfa1 (a.k.a. Byr4) is identified as a new component of the spindle damage checkpoint, which is resolved into two branches, one for metaphase arrest based on Mps1 and Mad1/2, the other for late anaphase arrest based on Bub2 and Bfa1. Bub2 and Bfa1 are localized to the spindle pole body.

49. Furge KA, Wong K, Armstrong J, Balasubramanian M, Albright CF: **Byr4 and Cdc16 form a two-component GTPase-activating protein for the Spg1 GTPase that controls septation in fission yeast**. *Curr Biol* 1998, **8**:947-954.

50. Shirayama M, Zachariae W, Ciosk R, Nasmyth K: **The Polo-like kinase Cdc5p and the WD-repeat protein Cdc20p/Fizzy are regulators and substrates of the anaphase promoting complex in *Saccharomyces cerevisiae***. *EMBO J* 1998, **17**:1336-1349.

51. Fesquet D, Fitzpatrick PJ, Johnson AL, Kramer KM, Toyn JH, Johnston LH: **A Bub2p-dependent spindle checkpoint pathway regulates the Dbf2p kinase in budding yeast**. *EMBO J* 1999, **18**:2424-2434.

Activation of the Dbf2 kinase occurs after activation of the Cdc5 kinase in the mitotic exit pathway and coincides with the onset of Clb2 degradation. Dbf2 kinase activity is repressed in cells arrested by the spindle checkpoint in a manner that depends on Bub2, but not Bub3 or Mad2. Whereas *bub2* and *mad2* single mutants arrest transiently in response to spindle damage, *bub2 mad2* double mutants fail to do so, suggesting the existence of two parallel branches of the spindle checkpoint pathway.

52. Komarnitsky SI, Chiang YC, Luca FC, Chen J, Toyn JH, Winey M, Johnston LH, Denis CL: **DBF2 protein kinase binds to and acts through the cell cycle-regulated MOB1 protein**. *Mol Cell Biol* 1998, **18**:2100-2107.

53. Fraschini R, Formenti E, Lucchini G, Piatti S: **Budding yeast Bub2 is localized at spindle pole bodies and activates the mitotic checkpoint via a different pathway from Mad2**. *J Cell Biol* 1999, **145**:979-991.

Bub2 and Mad1/2 act in parallel branches of the spindle checkpoint pathway as evidenced by the inability of *bub2 mad1* or *bub2 mad2* mutants to enforce cell cycle arrest. Furthermore, Apc26 function is required to eventually bypass the Mad2-dependent arrest but not the Bub2-dependent arrest. Bub2 is localized to the spindle pole body.

54. Visintin R, Hwang ES, Amon A: **Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus**. *Nature* 1999, **398**:818-823.

Cfi1 (a.k.a. Net1) is isolated as a Cdc14 interacting protein. Overexpression of *CFI1* causes late anaphase arrest with high levels of Clb2-Cdc28 kinase activity, while *cfi1* mutants are defective in Clb2-Cdc28 activation. Cfi1 localizes with Cdc14 to the nucleolus, except during a brief window in telophase when Cdc14 is released into the nucleus and the cytoplasm. Nucleolar localization of Cdc14 depends on Cfi1. Deletion of *CFI1* bypasses the requirement for Tem1, Dbf2, Cdc5, and Cdc15, suggesting that the sole function of the mitotic exit pathway is to disengage Cdc14 from Cfi1.

55. Shou W, Seol JH, Shevchenko A, Baskerville C, Moazed D, Chen ZW, Jang J, Charbonneau H, Deshaies RJ: **Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex**. *Cell* 1999, **97**:233-244.

An allele of *NET1* (a.k.a. *CFI1*) is isolated as a suppressor of a *cdc15* deletion, and also bypasses a *tem1* deletion. Net1 associates with several proteins including Cdc14 and Sir2, implicating Net1 in the control of mitotic exit and silencing, respectively. Net1 inhibits the phosphatase activity of Cdc14 *in vitro*.

56. Charles JF, Jaspersen SL, Tinker-Kulberg RL, Hwang L, Szidon A, Morgan DO: **The Polo-related kinase Cdc5 activates and is destroyed by the mitotic cyclin destruction machinery in *S. cerevisiae***. *Curr Biol* 1998, **8**:497-507.

57. Straight AF, Shou W, Dowd GJ, Turck CW, Deshaies RJ, Johnson AD, Moazed D: **Net1, a Sir2-associated nucleolar protein required for rDNA silencing and nucleolar integrity**. *Cell* 1999, **97**:245-256.

Net1 interacts with Sir2 and tethers Sir2 to rDNA in the RENT complex. Net1 is specifically required for rDNA silencing. At the end of mitosis a fraction of Sir2 redistributes to the nucleus suggesting a possible connection between rDNA silencing and the cell cycle.

58. Hwang LH, Lau LF, Smith DL, Mistrot CA, Hardwick KG, Hwang ES, Amon A, Murray AW: **Budding yeast Cdc20: a target of the spindle checkpoint**. *Science* 1998, **279**:1041-1044.

59. Cohen-Fix O, Koshland D: **The anaphase inhibitor of *Saccharomyces cerevisiae* Pds1p is a target of the DNA damage checkpoint pathway**. *Proc Natl Acad Sci USA* 1997, **94**:14361-14366.

60. Tinker-Kulberg RL, Morgan DO: **Pds1 and Esp1 control both anaphase and mitotic exit in normal cells and after DNA damage**. *Genes Dev* 1999, **13**:1936-1949.

A stabilized version of Pds1 prevents both anaphase and mitotic exit. Pds1 is also required to prevent Clb2 degradation and mitotic exit in the metaphase arrest induced by DNA damage. Clb2 degradation is delayed in *esp1* mutants but, in contrast to [61\*], is not further delayed by overexpression of stabilized Pds1. Sustained overexpression of *ESP1* leads to Clb2 degradation in cells arrested by nocodazole or DNA damage, suggesting that Pds1 may block mitotic exit by inhibiting Esp1.

61. Cohen-Fix O, Koshland D: **Pds1 of budding yeast has dual roles: inhibition of anaphase initiation and regulation of mitotic exit**. *Genes Dev* 1999, **13**:1950-1959.

A stabilized version of Pds1 blocks Clb2 degradation and cytokinesis, and prevents Cdh1 dephosphorylation. Clb2 degradation is delayed in *esp1* mutants but in contrast to [60\*] is abrogated altogether by expression of stabilized Pds1, leading to the proposal that Pds1 blocks Clb2 degradation and cytokinesis by a mechanism independent of Esp1.

62. Lim HH, Goh PY, Surana U: **Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast**. *Curr Biol* 1998, **8**:231-234.

63. Cross FR, Yuste-Rojas M, Gray S, Jacobson MD: **Specialization and targeting of B-type cyclins**. *Mol Cell* 1999, **4**:11-19.

Expression of *CLB2* from the *CLB5* promoter is unable to rescue the specialized function of Clb5 in DNA replication. Despite expression from the same *CLB5* promoter, Clb5 is degraded earlier than Clb2, consistent with the notion that Clb5 is an APC<sup>Cdc20</sup> substrate, while Clb2 is an APC<sup>Cdh1</sup> substrate. A potential Clb5 substrate-targeting domain is required for Clb5 function *in vivo*, but not for activation of Cdc28 *in vitro*.

64. Kaiser P, Moncollin V, Clarke DJ, Watson MH, Bertolaet BL, Reed SI, Bailly E: **Cyclin-dependent kinase and Cks/Suc1 interact with the proteasome in yeast to control proteolysis of M-phase targets**. *Genes Dev* 1999, **13**:1190-1202.

First, during virus infection, suicide of infected host cells thwarts viral replication and hence limits virus spread. Second, in nutrient-deprived conditions, altruistic cell suicide can ensure that the few remaining cells have sufficient substrate for continued survival. Finally, perhaps after genomic injury (e.g. caused by oxidative stress, irradiation, or alkylating reagents), certain pressure exists to transmit only properly replicated copies of the original ancestral genome rather than giving rise to clones whose altered genes could compete with the original founding genome [8–10].

### Yeast and apoptosis

The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Shizosaccharomyces pombe* have been employed extensively as models for genetic analysis of a variety of complex pathways and processes, including cell division, secretion, transcription, and receptor-mediated signal transduction. The great facility with which it is possible to genetically manipulate these single cell organisms makes yeast ideal for applying genetic approaches to many biological questions. But, what about the question of apoptosis? Do yeast possess cell suicide mechanisms that resemble apoptosis? The answer to this query remains controversial, but an apoptosis-like phenomenon has been described in *cdc48* mutants [11,12\*]. The CDC48 protein of *S. cerevisiae* is a putative ATPase that is normally involved in membrane fusion events associated with cell division and secretion. A particular temperature-sensitive (ts) mutant of *cdc48*, at the non-permissive temperature, results in nuclear fragmentation and membrane blebbing reminiscent of apoptosis seen in animal cells [11]. However, evidence is lacking that the apoptosis-like changes brought about by this ts-mutant of *cdc48* are anything more than coincidence. For example, it has not been shown that anti-apoptotic proteins from higher eukaryotes can rescue ts-*cdc48* strains from self-destruction.

### Ectopic expression of apoptosis protein in yeast

Ectopic expression in either *S. cerevisiae* or *S. pombe* of a variety of apoptosis-regulatory proteins derived from animal species has produced phenotypes that can be exploited for a variety of purposes in apoptosis research.

### Bcl-2 family proteins

The Bcl-2 family proteins play a pivotal role in apoptosis regulation, and consist of apoptosis-promoting members, such as Bax and Bak, as well as apoptosis-blockers including Bcl-2 and Bcl-X<sub>L</sub> (reviewed in [13–15]).

#### Apoptosis-inducing Bcl-2 family proteins

Sato *et al.* [16] were the first to recognize that ectopic expression of Bax confers a lethal phenotype in budding yeast. Since then, a variety of subsequent studies have confirmed and extended these observations to Bax homologs such as Bak and similar lethal phenotypes were also reported in fission yeast [17,18] (see Table 1). The cell death resulting from expression of Bax in yeast is suppressible by anti-apoptotic Bcl-2 family proteins,

**Table 1**

#### Comparison of Bax-induced cell death phenotype in mammalian cells and yeast.

Characteristics	Yeast	Mammals
Membrane blebbing	Yes	Yes
Chromatin condensation	Yes	Yes
DNA breakage	Yes	Yes
Caspase	Independent	Dependent and independent
Mitochondrial targeted	Yes	Yes
Cytchrome c release from mitochondria	Yes	Yes
Protection by Bcl-2	Yes	Yes
Pore-formation-domain dependency	Fully dependent	Partially dependent
Heterodimerization with endogenous Bcl-2 proteins	No	Yes

including Bcl-2 and Bcl-X<sub>L</sub>, but not by mutants of these proteins, which lack anti-apoptotic activity in mammalian cells (e.g. BH4 domain [N-terminal  $\alpha$ -helix]-deletion mutant and pore-forming domain deletion mutant) [19,20\*,21\*]. Bax and other apoptosis-inducing Bcl-2 family proteins are known to have two ways to induce mammalian cell death. One is through heterodimerization with anti-apoptotic Bcl-2 family proteins (e.g. Bcl-2) suppressing their cytoprotective activity. The other is through its own intrinsic cytotoxic activity, probably by forming ion-channels or pores in membranes, as described later. Similarly, cytoprotective Bcl-2 family proteins prevent cell death via heterodimerization-dependent and -independent mechanisms. In yeast cells, dimerization of Bcl-2/BclXL with Bax is not necessary for suppression of Bax-induced cell death, a finding that is similar to results derived from animal cell experiment [21\*,22,23]. Bax is localized predominantly to mitochondria when expressed in budding yeast [24] and induces mitochondrial membrane hyperpolarization [21\*] and release of cytochrome c from these organelles [25], much the same as in higher eukaryotes (reviewed in [7\*]). Thus, some of the mechanisms used by Bax for promoting cell death appear to be conserved from yeast to humans. However, differences also exist, since some mutants of Bax, which are inactive in yeast, retain ability to kill mammalian cells, probably by dimerizing with endogenous anti-apoptotic Bcl-2 family proteins [20\*]. In cells lacking endogenous Bcl-2 homologs, such as yeast cells, Bax is deprived of its dimerization-dependent mechanism for inducing cell death, and instead is limited to whatever intrinsic cytotoxic functions it possesses. The lethal phenotype conferred by Bax in yeast provides an ideal opportunity to study dimerization-independent mechanisms of Bax. In addition, since Bax is known to activate both caspase-dependent and -independent cell death cascades in mammalian cells [26], the absence of caspases in yeast also provides system in which to interrogate caspase-independent cell death mechanism employed by Bax.

Although the molecular explanation for Bax-induced cell death in yeast remains controversial, presumably it can be attributed to the predicted structural similarity of Bax with the pore-forming domains of certain bacterial toxins, such as the colicins and diphtheria toxin (reviewed in [27]). Bax forms channels in synthetic membranes *in vitro* [20•,28,29]. Deletion of the putative pore-forming  $\alpha$ 5 and  $\alpha$ 6 helices in Bax abolishes its cytolytic activity in yeast and its channel activity *in vitro* [20•]. Although far from proven, it is speculated that Bax forms pores directly in mitochondrial membranes or that it collaborates with other channel proteins to alter membrane potential and permit cytochrome c release [29,30•,31•]. Consistent with this idea, a subgroup of pro-apoptotic Bcl-2 family proteins that lack the putative pore-forming domain have proven uniformly inactive (not lethal) when expressed in yeast (e.g. Bcl-X<sub>S</sub>, BAD) [16,32]. These functions of Bax observed in yeast may be conceptually similar to the cytotoxic mechanisms employed by the bacterial colicins, a family of pore-forming proteins produced by bacteria. Colicins are secreted by particular strains of bacteria as soluble proteins, which bind receptors on competing bacteria and kill them by forming lethal pores that depolarize bacterial membranes, resulting in cell death (reviewed in [33]). Protection from self-destruction of colicin-secreting strains is ensured by immunity proteins, which bind colicins and prevent their membrane translocation and pore-formation [34]. Given that mitochondria originated from eubacteria, which formed a symbiotic relation with host eukaryotic cells (reviewed in [7•]), a reasonable speculation is that some aspects of cell death mechanisms employed by Bax evolved from bacterial systems and were adapted for cell suicide responses by higher eukaryotes.

#### Apoptosis-blocking Bcl-2 family proteins

While Bax induces yeast cell death, ectopic expression of the anti-apoptotic protein Bcl-2 can protect yeast in certain circumstances. Strains of yeast deficient in superoxide dismutase (SOD) are able to grow better in oxygenated environments and survive longer when placed into water (osmotic stress) when expressing human Bcl-2 [35,36]. How Bcl-2 performs its cytoprotective mission in yeast remains unclear. Conceivably, this may be attributable to Bcl-2's predicted structural similarity to channel-forming proteins [37] and its ion-channel activity [38]. Deletion of the putative pore forming  $\alpha$ 5– $\alpha$ 6 region of Bcl-2 and omission of the carboxy-terminal membrane-anchoring domain required for mitochondrial-targeting abrogates Bcl-2's ability to protect against Bax-induced cell death in yeast [19,20•]. However, further structure–function analysis of the Bcl-2 protein with respect to domains necessary for cytoprotection in yeast is needed to provide more insights into its mode of action.

Nevertheless, yeast has proven a convenient system for performing rapid functional analysis of Bcl-2 and Bcl-X<sub>L</sub> mutants [19,20•,21•,22,23]. Without exception, mutations that negate the ability of Bcl-2 or Bcl-X<sub>L</sub> to rescue yeast against Bax have thus far resulted in loss-of-function phenotypes when tested

in mammalian cells. Thus, a conservation of mechanisms employed by Bcl-2/Bcl-X<sub>L</sub> for promoting cell survival seems to exist across wide evolutionary distances, a concept consistent with the presence of Bcl-2 homologs in both invertebrate and vertebrate animal species [39]. This does not, however, exclude additional functions for the Bcl-2 or Bcl-X<sub>L</sub> proteins in higher eukaryotes, where binding to diverse proteins involved in signal transduction, transcription, and caspase regulation has been reported (reviewed in [13,14]). It does imply, however, that certain core-functions of these anti-apoptotic Bcl-2 family proteins exist and that yeast may be a useful system for focusing on these.

#### Yeast-based functional screening of new regulators of Bcl-2 family proteins

The ability of Bax to confer a lethal phenotype in yeast has permitted exploitation of the advantage of yeast genetics for gaining improved understanding of the apoptosis pathway, including high-copy suppressor screens, identification of yeast genes essential for Bax-lethality by complementation cloning, and targeted gene ablation. For example, human cDNA libraries in yeast expression plasmids have been used to screen for suppressors of Bax, resulting in the identification of Bax inhibitor-1 (BI-1) a multiple membrane-spanning protein. In mammalian cells, BI-1 associates with protein complexes containing Bcl-2 and Bcl-X<sub>L</sub>, protects against apoptosis induced by Bax and certain other stimuli, and is required for cell survival [40••]. Two-hybrid screening of Bak-associating proteins in *S. pombe* cDNA library resulted in the finding of Calnexin homolog Cnx1 as an endogenous enhancer for Bak-induced lethality in *S. pombe*, although the functional significance of Calnexin for regulation of mammalian apoptosis has not yet been well characterized [41].

Classic yeast genetics approaches were additionally used to generate Bax-resistant mutant strains, which were exploited in complementation cloning strategies, resulting in the discovery that components of the mitochondrial FoF1-ATPase/H<sup>+</sup> pump, which are conserved from yeast to human, are required for Bax-lethality in yeast [42••]. Subsequent experiments using oligomycin, a FoF1-ATPase inhibitor, provided evidence that the H<sup>+</sup>-pump is also required for Bax-induced apoptosis in mammalian cells. This finding illustrates an advantage of working with yeast for studying mitochondrial involvement in cell death — namely, because yeast are readily capable of anaerobic growth (unlike mammalian cells), a wide variety of nuclear genes encoding mitochondrial proteins can be knocked-out without lethality. For example, targeted gene ablation approaches were employed to demonstrate the requirement of the voltage-dependent anion channel (VDAC) and adenine nucleotide translocator (ANT) for Bax-lethality in yeast, thus corroborating biochemical data which suggested physical and functional interactions of Bcl-2 family proteins with these mitochondrial proteins [30•,31•,43]. Interestingly, VDAC and ANT are components of the mitochondrial permeability transition

pore-complex, a multiprotein structure that has been implicated in apoptosis induced by myriad stimuli (reviewed in [44]).

#### CED-4

The CED-4 protein plays an essential role in developmental PCD in the nematode *Caenorhabditis elegans* [45]. CED-4 contains a caspase-binding domain, CARD (caspase recruitment domain) [46] and an ATP-binding domain, which is predicted to permit oligomerization of this protein. Oligomerization of CED-4 brings the associated pro-caspases into close proximity and results in their trans-proteolysis to generate the processed and autonomously active proteases [47]. CED-4 homologs have been identified in mammalian cells [48,49] and insects [50,51], suggesting that CED-4 family proteins play key roles in apoptosis regulation of higher eukaryotes. Ectopic expression of CED-4 in *S. pombe* [52] and *S. cerevisiae* [32] is lethal, whereas ATP-binding site (P-loop) mutants of CED-4 are not [32,52]. Moreover, CED-9, the worm homolog of Bcl-2, binds CED-4 and rescues yeast from lethal effect of CED-4 expression [32,52]. However, given the absence of caspases in yeast, it is unclear whether this phenotype of CED-4 is physiologically relevant. Moreover, unlike animal cells in which CED-4 is cytosolic, CED-4 accumulates in the nuclei of fission yeast. Nevertheless, CED-4-mediated lethality in fission yeast could provide the basis for a convenient assay for cDNA library screening in search of proteins that modify the function of this protein.

#### Caspases

Ectopic expression of active caspases in yeast can result in either death or growth arrest, depending on the caspase and the levels of expression [53•,54•,55,56•,57•]. Since yeast have no endogenous caspase-like proteases, caspase-induced cell death and growth inhibition may be non-physiological phenomena. Nevertheless this property is exploitable for studying caspases and caspase-inhibitory proteins. Caspase-cleavable reporters have also been generated for monitoring activity of these proteases in yeast [53•]. Caspases are initially synthesized as single polypeptide chains representing the inactive zymogens. Activation generally requires proteolytic processing, generating the large and small subunits of the active enzyme from this pro-protein (reviewed in [3]). Thus, some efforts to reconstitute active caspases in yeast have relied upon expressing the large and small subunits from separate plasmids [55]. Alternatively, recognizing that the amino and carboxyl termini of many proteins are located in close proximity when their three-dimensional structures are analyzed, the technique of circular permutation has been employed for expressing single polypeptide chains that begin at the cleavage site normally located between the large and small subunits, and fold to yield active proteases without requirement for proteolytic processing [54•,57•]. These modified caspases were used to study interactions of a *Drosophila* inhibitor of apoptosis protein (IAP) with caspases, resulting in rescue of yeast

from the lethal effects of ectopic expression of an active caspase [54•]. Co-expression of the IAP-binding *Drosophila* protein Hid resulted in restoration of caspase activity and killing of yeast, thus providing evidence that Hid functions as a repressor of IAPs, which in turn repress caspases [54•]. A clever application of the yeast two-hybrid technique has also been described for identifying caspase substrates encoded by cDNA libraries [58••].

#### Conclusions

Although yeast lack caspases and therefore do not possess the same apoptotic pathway recognized in animal species, these simple unicellular eukaryotes can be exploited in a wide variety of ways for apoptosis research. First, yeast can be used for structure-function (mutagenesis) analysis of apoptosis-regulating protein when a phenotype is available. Second, two-hybrid and related protein interaction cloning strategies have been developed. Third, functional screens have been used for identification of cDNAs that suppress apoptosis protein-induced lethality. Fourth, genetic analysis of apoptosis-regulating protein interactions with host proteins has been studied using targeted gene ablation. Finally, classic complementation cloning has identified yeast genes required for phenotypes conferred by ectopic expression of animal apoptosis regulators. It is imperative, however, that observations derived from initial analysis in yeast be confirmed through experimentation in appropriate animal cells. The experience thus far has been that, in many instances, discoveries made originally in yeast have been experimentally validated in higher eukaryotic cells, suggesting that yeast-based strategies for studying apoptosis genes will continue to provide novel insights into conserved cell death mechanisms. Perhaps equally important, yeast-based functional assays for human apoptosis proteins also provide powerful systems for high through-put drug screening. Exploiting these assays for small-molecule compounds that alter apoptosis proteins in clinically useful ways, therefore, may ultimately become one of the greatest contributions that yeast make to humans — besides bread and wine.

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# Motility in *Myxococcus xanthus* and its role in developmental aggregation

Mandy J Ward and David R Zusman

The Frz signal transduction system of *Myxococcus xanthus* was originally thought to be a simple variation of the well-characterized Che system of the enteric bacteria. Recently, however, many additional Frz proteins, along with alternative signal transduction systems, have been discovered. Together these signal transduction pathways coordinate cell-cell behavior, permitting the complex interactions required for developmental aggregation and fruiting body formation.

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## Abbreviations

ABC	ATP-binding cassette
HPt	histidine-containing phosphotransfer
MCP	methyl-accepting chemotaxis protein
PE	phosphatidylethanolamine

## Introduction

The study of social interactions within bacterial populations has elicited much excitement in recent years after the identification of quorum sensing in a wide range of bacterial species [1]. However, cell-density-dependent behavior was observed over a century ago in the myxobacteria by Thaxter [2]. Under starvation conditions, the myxobacteria undergo a spectacular morphogenesis during which tens of thousands of cells interact and form complex aggregates, which mature into fruiting bodies containing spores. The process of fruiting body formation requires the cells to be actively motile on a solid surface and present at a high cell density. In this review, we discuss the relationship between motility and extracellular signaling in *Myxococcus xanthus*, the most extensively studied of the myxobacteria, and speculate on how cell-cell communication can result in the formation of complex multicellular structures.

## *M. xanthus* moves by gliding motility

Gliding motility has been defined as the smooth movement of a cell on a surface in the direction of the long axis of the cell, without the requirement for flagella (see <http://www.microbiology.med.umn.edu/myxobacteria/index.html> for time-lapse movies of various types of gliding motility). The mechanism of gliding motility is poorly understood and, in fact, *M. xanthus* has two genetically distinct motility systems, which together facilitate wild-type movement [3,4].

## The adventurous A-motility system

A-motility allows the movement of both isolated single cells and cells in groups, and is most effective on relatively

firm surfaces [5]. Cells with mutations in A-motility genes (A-S<sup>+</sup>) can still swarm on soft agar and many can still form fruiting bodies [6]. However, these mutants are defective in following lines of stress in agar (elasticotaxis) [7]. Little is known about the mechanism of A-motility, and only a single gene (*cglB*) from over 30 A-motility loci (*agl* and *cgl* genes) has been sequenced to date [8]. Sequence analysis of the *cglB* gene suggests that CglB is an outer-membrane lipoprotein, but its role in A-motility remains unknown.

## The social S-motility system

Cells gliding using only the social motility system (A-S<sup>+</sup>) form smooth-edged colonies, since single cells are unable to move away from the swarm. Kaiser [9] first suggested that a specific organelle, the type IV pilus, might be required for S-motility. Expression of the *pilA* gene, which encodes the prepilin subunit, is controlled by a two-component signal transduction system, encoded by the *pilS* and *pilR* genes [10,11]. The PilA prepilin is processed by the PilD peptidase, which also methylates the amino terminus of the mature pilin subunits. Polymerization of the pilus requires the PilB, C, M, N, O and P proteins, while the PilQ protein (formerly SglA) acts in the role of a secretin, forming a channel in the outer membrane [12]. Cells with mutations in the *pilT* gene are pilated, although unable to move by social motility [13]. The PilT protein has, therefore, been proposed to be essential for the activity of the pilus and perhaps even to play the role of a motor protein [14\*].

Interestingly, all of the above components (PilA, B, C, D, M, N, O, P, Q, S, R and T) have homologues in the type IV pilus systems of *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*, which are involved in another surface-associated translocation mechanism termed twitching motility [15]. Bradley [16] proposed what is thought to be the most likely mechanism for both twitching and S-motility: the extrusion and subsequent retraction of the pili, which could generate force against either the substratum or another cell. It should be noted, however, that there are several differences between S-motility and twitching. These differences include the presence of three genes, *pilG*, *pilH* and *pili*, which together encode an ATP-binding cassette (ABC) transporter system found only in *M. xanthus* [17,14\*].

Several other genes have also been shown to be required for S-motility. Cells with mutations in the *tgl* gene do not have polar type IV pili, but can be transiently stimulated to produce pili and move after contact with other nonmutant *tgl* cells [18]. Like CglB, Tgl appears to be a lipoprotein [19]. Cells with mutations in *dsp* are defective in S-motility. The *dsp* gene is required for the production of an extracellular polysaccharide/protein (IFP-1) matrix termed fibrils [20–22]. SglK, which shows similarity to the molecular

# The Mitochondrial $F_0F_1$ -ATPase Proton Pump Is Required for Function of the Proapoptotic Protein Bax in Yeast and Mammalian Cells

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## Summary

The proapoptotic mammalian protein Bax associates with mitochondrial membranes and confers a lethal phenotype when expressed in yeast. By generating Bax-resistant mutant yeast and using classical complementation cloning methods, subunits of the mitochondrial  $F_0F_1$ -ATPase proton pump were determined to be critical for Bax-mediated killing in *S. cerevisiae*. A pharmacological inhibitor of the proton pump, oligomycin, also partially abrogated the cytotoxic actions of Bax in yeast. In mammalian cells, oligomycin also inhibited Bax-induced apoptosis and activation of cell death proteases. The findings imply that an intact  $F_0F_1$ -ATPase in the inner membrane of mitochondria is necessary for optimal function of Bax in both yeast and mammalian cells.

## Introduction

Altered function of mitochondria has been recognized for many years as an important contributor to ischemic and necrotic cell death (Bernardi et al., 1994). Recently, however, evidence has accumulated suggesting a critical role for these organelles in apoptosis and programmed cell death (Petit et al., 1996; Hirsch et al., 1997).

Proteins of the Bcl-2 family are important regulators of mammalian cell life and death, with some functioning to prevent and others to promote apoptosis (Reed, 1994; Yang and Korsmeyer, 1996). These proteins can also modulate cell death processes that result in necrotic rather than apoptotic cell death, under some circumstances (Kane et al., 1995; Shimizu et al., 1996). Most Bcl-2 family proteins are integral membrane proteins that reside in the outer mitochondrial membrane, as well as some other intracellular membranes (Krajewski et al., 1993; González-García et al., 1994).

At present, the biochemical mechanism by which Bcl-2 and its homologs regulate cell death remains controversial (Reed, 1997). The three-dimensional structure of one of the Bcl-2 family proteins suggests similarity to the pore-forming domains of certain bacterial toxins, such as diphtheria toxin and the colicins (Muchmore et al., 1996). Moreover, the antiapoptotic proteins Bcl-2

and Bcl-X<sub>L</sub> as well as the proapoptotic protein Bax can form ion channels in synthetic membranes *in vitro* in a pH-dependent manner (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). However, Bcl-2 and Bcl-X<sub>L</sub> also clearly have apoptosis-regulatory functions apart from their ability to form channels, in that they bind to several other proteins that can modulate responses to apoptotic stimuli (Reed, 1997). Bcl-2 has been shown to protect mitochondria from loss of membrane potential and release of caspase-activating proteins such as cytochrome c and apoptosis-inducing factor (AIF) (Susin et al., 1996; Zamzami et al., 1996; Kluck et al., 1997; Yang et al., 1997), whereas Bax can induce loss of mitochondrial membrane potential and activation of caspases (Xiang et al., 1996; Jürgensmeier et al., 1997). It remains unclear whether these effects of Bcl-2 and Bax on mitochondrial physiology are a direct result of their intrinsic activities as channel proteins, which conceivably may transport either ions or proteins, as opposed to an indirect consequence of their effects on other channel proteins associated with mitochondrial membranes.

When expressed in either the budding yeast *Saccharomyces cerevisiae* or the fission yeast *Schizosaccharomyces pombe*, the proapoptotic mammalian protein Bax confers a lethal phenotype (Sato et al., 1994; Ink et al., 1997; Jürgensmeier et al., 1997). Yeast cells undergoing Bax-induced death exhibit ultrastructural changes that include massive cytosolic vacuolarization and apparent disruption of mitochondria (Ink et al., 1997; Jürgensmeier et al., 1997), similar to mammalian cells that express Bax in the presence of caspase inhibitors (Xiang et al., 1996). In yeast, the Bax protein is associated primarily with mitochondria, and the targeting of Bax to these organelles appears to be important for its lethal phenotype in yeast (Zha et al., 1996). Similar to mammalian cell apoptosis, expression of Bax in yeast has been reported to induce release of cytochrome c from mitochondria into the cytosol (Manon et al., 1997). Yeast cell death induced by Bax or the closely related Bak protein can be specifically suppressed by antiapoptotic Bcl-2 family proteins (Sato et al., 1994; Bodrug et al., 1995; Hanada et al., 1995; Greenhalf et al., 1996; Zha et al., 1996; Ink et al., 1997; Jürgensmeier et al., 1997). Further evidence of similarities in at least some of the mechanisms by which Bax functions in animal cells and yeast comes from the observation that cell death induced by Bax can be suppressed in both mammalian cells and *S. cerevisiae* by BI-1, a human protein that contains multiple membrane-spanning domains (Xu and Reed, 1998 [this issue of *Molecular Cell*]).

The functions of Bcl-2 family proteins are often conserved across evolution, with the human Bcl-2 protein, for example, exhibiting potent antiapoptotic activity even in nematodes and insect cells (Alnemri et al., 1992; Vaux et al., 1992; Hengartner and Horvitz, 1994). Moreover, the human Bcl-2 protein has been reported to protect superoxide dismutase (sod)-deficient strains of budding yeast from cell death induced by oxidative stress (Kane et al., 1993), implying an evolutionarily

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conserved function perhaps even in some unicellular organisms. Though programmed cell death (PCD) is conventionally thought to operate only in multicellular organisms, recent studies have revealed apoptosis-like cell death in several unicellular eukaryotes, including *Dictyostelium discoideum* (Cornillon et al., 1994), *Trypanosome brucei rhodesiense* (Welburn et al., 1996), *Trypanosome cruzi* (Ameisen et al., 1996), *Leishmania amazonensis* (Moreira et al., 1996), and *Tetrahymena thermophila* (Christensen et al., 1995). It has also been suggested that certain forms of PCD may even exist in prokaryotes (Ameisen, 1996), where cell suicide mechanisms could potentially limit spread of viruses, reduce competition for nutrients during times of starvation, or ensure that cells with damaged DNA do not pass their defective genomes on to future generations. Reasoning that at least some of the functions of Bcl-2 family proteins appear to be conserved in yeast, we undertook a classical genetics approach designed to identify yeast genes that are required for Bax-mediated lethality in *S. cerevisiae*.

## Results

### Creation of a Mutant Yeast Strain that Displays

#### Resistance to Bax-Induced Cell Death

Yeast strain EGY48 was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (MMNG) and then transformed with YEpl51-Bax, a *LEU2*-marked, high-copy episomal plasmid that produces the mouse Bax protein under the control of the galactose-inducible *GAL10* promoter (Zha et al., 1996). Transformants were plated on leucine-deficient semisolid medium containing galactose, resulting in ~50 viable colonies. To exclude clones that might have survived because of defects in transactivation of the *GAL10* promoter in YEpl51-Bax, these transformants were cured of the YEpl51-Bax plasmid and then retransformed with the plasmid pEG202-Bax in which Bax is produced from a strong constitutive *ADH1* promoter. The resulting 24 Bax-resistant clones were mated with wild-type Myy290 strain cells, yielding 9 diploids in which sensitivity to Bax-mediated killing was restored, thus suggesting a recessive mutation. Tetrad analysis was then performed for these recessive mutants, with only one (hereafter designated as Bax-resistant mutant-1 [BRM1]) exhibiting 2:2 Mendelian segregation of the Bax-resistance phenotype in a manner consistent with a single gene defect (Figure 1A).

The genomic mutation in BRM1 cells did not interfere with Bax protein production, as determined by immunoblotting (Figure 1B). For these experiments, the wild-type and BRM1 cells were transformed with pGilda-Bax, which produces Bax as a fusion protein with a portion of LexA (used analogous to an epitope tag here), or the pGilda plasmid, which produces only the LexA fragment. The addition of the LexA tag to Bax does not interfere with its intracellular targeting or cell death-inducing function in yeast (Zha et al., 1996).

### Complementation Cloning of Yeast Genes that Restore Sensitivity to Bax

The BRM1 cells containing pGilda-Bax were transformed with a centromere-based yeast genomic library.

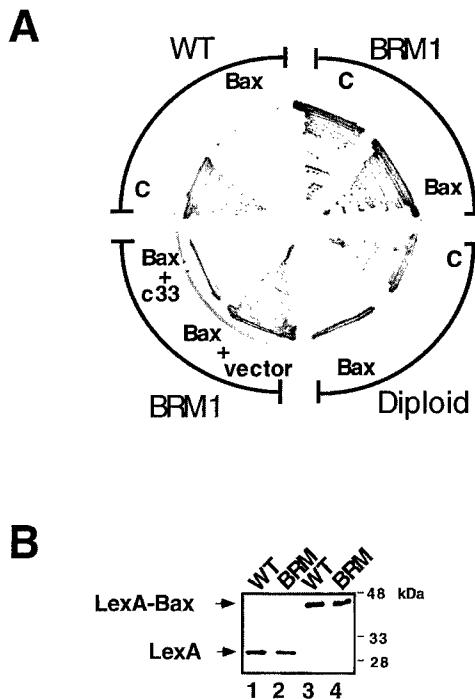


Figure 1. Generation of a Bax-Resistant Yeast Mutant and Identification of Genomic Clones that Restore Bax Sensitivity

(A) Wild-type EGY48 yeast (WT), Bax-resistant-mutant (BRM1), and diploid cells derived from mating Myy290 (wild-type)  $\times$  BRM1 were transformed with control plasmid pGilda (C) or galactose-inducible pGilda-Bax (Bax). BRM1 was also cotransformed with pGilda-Bax and clone 33 from yeast genomic library or control vector YCP50. Each transformant was first grown on glucose-based plate and then a colony was restreaked on galactose-containing plates and incubated at 30°C for 4 days.

(B) Immunoblot analysis is shown for lysates (10  $\mu$ g) derived from WT yeast (lanes 1 and 3) and BRM1 (lanes 2 and 4) cells transformed with pGilda (produces LexA protein DNA-binding domain without a nuclear localization sequence) (lanes 1 and 2) or pGilda-Bax (produces LexA-Bax fusion protein) (lanes 3 and 4). Antigens were detected using anti-LexA rabbit antiserum.

Eight transformants were identified by replica plating that appeared to have a restoration of their sensitivity to Bax-mediated cell death. The plasmids recovered from these eight transformants were then retransformed with pGilda-Bax into BRM1 cells, with only one of these clearly restoring sensitivity to Bax-mediated lethality to wild-type levels (Figure 1A). DNA sequence analysis revealed that this clone contained three genes, including *ATP4*, which encodes subunit 4 of the yeast  $F_0F_1$ -ATPase, a proton pump located in the inner membrane of mitochondria (Weber and Senior, 1997).

### ATP4 Is Required for Bax-Induced Lethality in Yeast

Since it has been previously suspected that Bax promotes cell death at least in part through effects on mitochondria (Xiang et al., 1996; Zha et al., 1996), we focused on *ATP4* as a likely candidate gene that is required for Bax-induced killing of yeast. The *ATP4* gene has been previously disrupted in *S. cerevisiae* by *URA3* insertional mutagenesis (Velours et al., 1989; Paul et al., 1992), thus

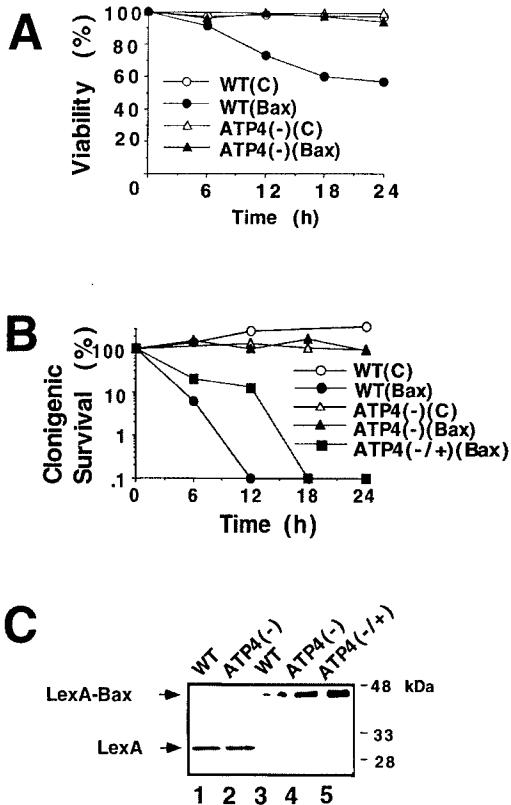


Figure 2. Yeast with Disrupted ATP4 Gene Are Resistant to Bax-Induced Cell Death

Wild-type D273-10B/A yeast, *ATP4* knockout yeast strain PVY10 (*ATP4*<sup>-</sup>), and heterozygous diploids derived from mating of PVY10 with Myy290 (*ATP4*<sup>-/+</sup>) were transformed with pGilda (C) or pGilda-Bax. The cells were grown in glucose-based medium and then recultured in galactose-based medium to induce protein expression from the *GAL1* promoter in pGilda plasmids.

(A) The percentage of trypan blue dye excluding cells was determined at various times after switching to galactose-based media (mean  $\pm$  SE;  $n = 3$ ; SE bars are obscured by symbols).

(B) Clonogenic survival was determined by recovering cells at various times from galactose-containing medium and plating 1000 cells on glucose-based semisolid medium. Data are representative of at least three experiments.

(C) Immunoblot analysis was performed to assess LexA and LexA-Bax protein levels in cells after 12 hr of culture in galactose-based media, as described for Figure 1.

creating the *ATP4*-deficient strain PVY10. We therefore tested PVY10 cells for resistance to Bax. For these experiments, PVY10 cells were transformed with pGilda-Bax or pGilda control plasmid and grown for various times in galactose-containing medium to induce expression of Bax, and cell viability was monitored by trypan blue dye exclusion. *ATP4*-deficient PVY10 cells were not killed after switching from glucose- to galactose-containing medium (Figure 2A). In contrast, cells of the isogenic wild-type strain (D273-10B/A) that had been transformed with pGilda-Bax began to die within 12 hr after switching to galactose.

To further verify that *ATP4*-deficient PVY10 cells are resistant to Bax-mediated lethality, a clonogenic survival assay was performed in which cells were switched from glucose- to galactose-containing liquid medium for 1

day to induce expression of Bax, and then plated on glucose-based semisolid medium, which suppresses the *GAL1* promoter in pGilda. The plating efficiency of *ATP4*-deficient PVY10 cells was essentially the same, regardless of whether they contained the pGilda-Bax or pGilda plasmids (Figure 2B). In contrast, colony formation by the isogenic wild-type strain was markedly reduced in cells harboring the pGilda-Bax plasmid compared to the control pGilda vector. Clonogenic survival of the wild-type cells began to decline after as little as 6 hr of exposure to galactose, with essentially all cells failing to form viable colonies after a 12 hr exposure. The differences in the kinetics of loss of clonogenic survival and development of trypan blue positivity (compare Figures 2A and 2B) have been observed previously in Bax-expressing yeast (Zha et al., 1996; Jürgensmeier et al., 1997), and likely reflect a commitment to cell death even before loss of plasma membrane integrity. Mating the PVY10 cells with wild-type haploids to create *ATP4*<sup>+/+</sup> heterozygous diploids restored sensitivity to Bax. Immunoblot analysis demonstrated that the *ATP4* mutation did not prevent production of Bax protein (Figure 2B). We conclude therefore that *ATP4*, which is a nuclear gene that encodes subunit 4 of the yeast mitochondrial  $F_0F_1$ -ATPase proton pump, is required for Bax-mediated killing of yeast.

#### The Proton-Pump Inhibitor Oligomycin Inhibits Bax-Induced Killing of Yeast

Oligomycin binds to the  $F_0$  portion of the yeast and mammalian  $F_0F_1$ -ATPases and prevents the proton pump from transporting  $H^+$  ions, thus effectively shutting it off (Tzagoloff, 1970). We reasoned that if the proton pump is required for Bax-mediated lethality in yeast, oligomycin should render wild-type yeast resistant to Bax. To test this hypothesis, D273-10B/A strain yeast that had been transformed with either pGilda-Bax or pGilda control plasmids were cultured for various times in galactose-containing medium with or without oligomycin. Oligomycin did not inhibit the growth of yeast under these conditions due to their ability to produce sufficient ATP from anaerobic fermentation (not shown). Oligomycin is non-toxic in yeast and it fails to induce permeability transition pore (PTP) opening in yeast mitochondria (Jung et al., 1997). As shown in Figure 3, oligomycin partially inhibited the Bax-induced killing of yeast, without interfering with production of the LexA-Bax protein. Thus, similar to disruption of the *ATP4* gene, a pharmacological inhibitor of the  $F_0F_1$ -ATPase proton pump suppresses Bax-mediated lethality in yeast.

#### Respiration Is Not Required for Bax-Induced Killing of Yeast

It was possible that the genetic disruption or pharmacological inhibition of the  $F_0F_1$ -ATPase proton pump indirectly suppressed Bax-mediated lethality in yeast by blocking respiration (Velours et al., 1989; Paul et al., 1992; Giraud and Velours, 1994). To address this question and to further explore the importance of the  $F_0F_1$ -ATPase, we compared the effects of Bax expression on the viability and clonogenic survival of *rho*<sup>-</sup> yeast as well as on an additional mutant strain of yeast in which the

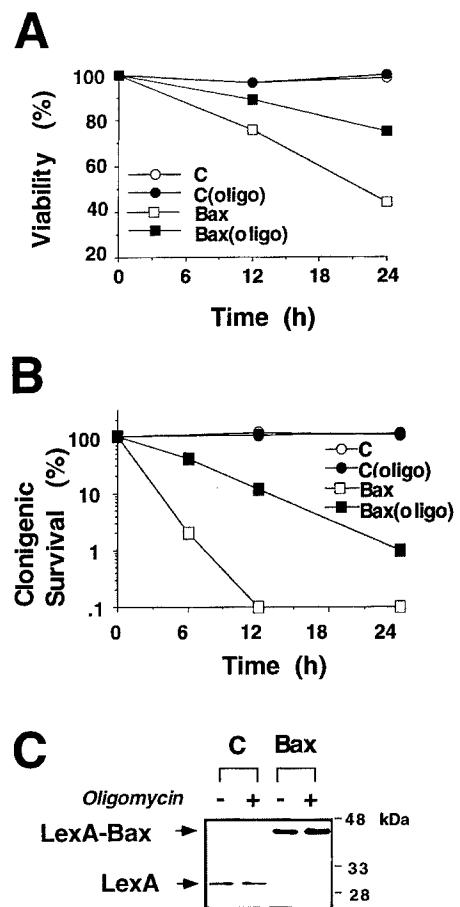


Figure 3.  $F_0F_1$ -ATPase Proton-Pump Inhibitor, Oligomycin, Attenuates Bax-Induced Cell Death in Yeast

Yeast strain D273-10B/A cells transformed with pGilda (C) or pGilda-Bax were initially cultured in glucose-based media, then switched to galactose-containing medium with (closed symbols) or without (open symbols) 10  $\mu$ M oligomycin, and the percentage of trypan blue dye excluding cells was determined at various times thereafter (A) or cells were recovered and either 1000 or 3000 cells were plated on glucose-containing semisolid medium (B) (mean  $\pm$  SE;  $n = 3$ ; SE symbols are obscured by symbols). In (C), protein lysates (10  $\mu$ g) were generated from the same cells after 12 hr of culture in galactose-based medium and analyzed by immunoblotting using anti-LexA antiserum. Lanes 1/2 and lanes 3/4 represent cells containing pGilda or pGilda-Bax, respectively, grown with or without oligomycin as indicated.

$\delta$  subunit of  $F_0F_1$ -ATPase had been inactivated by *URA3* insertional mutagenesis (Giraud and Velours, 1994). Unlike the *ATP4* mutant, both *rho*<sup>-</sup> and *ATP- $\delta$* -deficient yeast are *petites*. *Rho*<sup>-</sup> yeast fail to express all proteins encoded in the mitochondrial genome, and thus lack certain proteins that are critical for respiration. Though certain subchains of the  $F_0F_1$ -ATPase proton pump are encoded in the mitochondrial genome, its activity remains partially functional in *rho*<sup>-</sup> yeast (Schatz, 1968; Giraud and Velours, 1994). In contrast, loss of the nuclear-encoded *ATP- $\delta$*  protein results in deficient function of both the  $F_0$  and  $F_1$  components of the proton pump (Giraud and Velours, 1997). These two *petite* strains grew at comparable rates in the absence of Bax (not shown).

The *rho*<sup>-</sup> yeast transformed with pGilda-Bax began

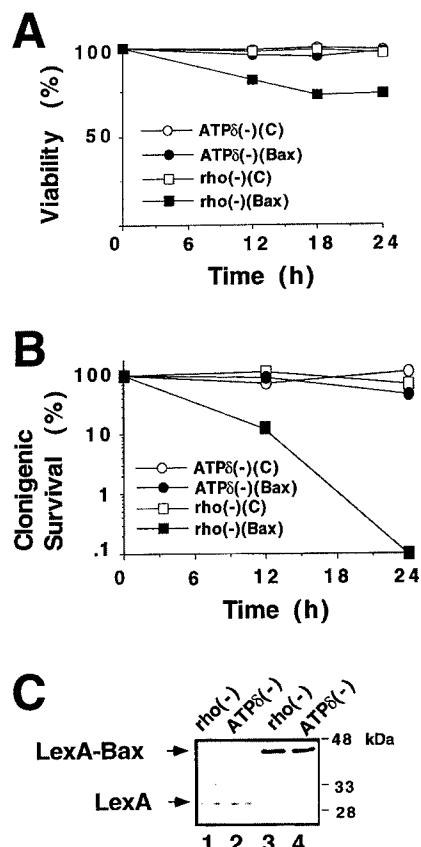


Figure 4. *ATP $\delta$* -Deficient but Not *rho*<sup>-</sup> petite Yeast Are Bax-Resistant

Strain CGY1 yeast that harbor a *URA3*-disrupted *ATP $\delta$*  gene and *rho*<sup>-</sup> yeast (both *petite*) were transformed with pGilda (C; open symbols) or pGilda-Bax (closed symbols). Cell viability (A), clonogenic survival (B), and Bax protein levels (C) were measured as described for Figure 2 at various times after switching cells to galactose-containing medium (mean  $\pm$  SE;  $n = 3$ ; some SE symbols are obscured by symbols). In (C), lanes 1/2 and lanes 3/4 represent *rho*<sup>-</sup> and *ATP $\delta$* -deficient yeast transformed with pGilda and pGilda-Bax, respectively.

to die when switched to galactose-containing medium, whereas *rho*<sup>-</sup> cells containing the control pGilda vector did not (Figure 4A). Note however that the Bax-induced cell death and loss of clonogenic survival occurred with delayed kinetics relative to wild-type yeast (compare with Figure 2). Thus, the absence of respiration in *rho*<sup>-</sup> cells may reduce but does not abrogate Bax-mediated lethality in yeast. In contrast, yeast lacking the  $\delta$  subunit of the  $F_0F_1$ -ATPase were completely resistant to Bax, despite expressing LexA-Bax protein at levels equivalent to those of the *rho*<sup>-</sup> cells (Figure 4). We conclude therefore that respiration is not required for Bax-induced killing of yeast, but the  $F_0F_1$ -ATPase proton pump is.

#### Oligomycin Also Inhibits Bax-Induced Apoptosis and Activation of Cell Death Proteases in Mammalian Cells

No mammalian cells exist that harbor mutations within subunits of the mitochondrial  $F_0F_1$ -ATPase. Thus, to explore whether the proton pump is also required for optimal function of Bax in mammalian cells, one is limited

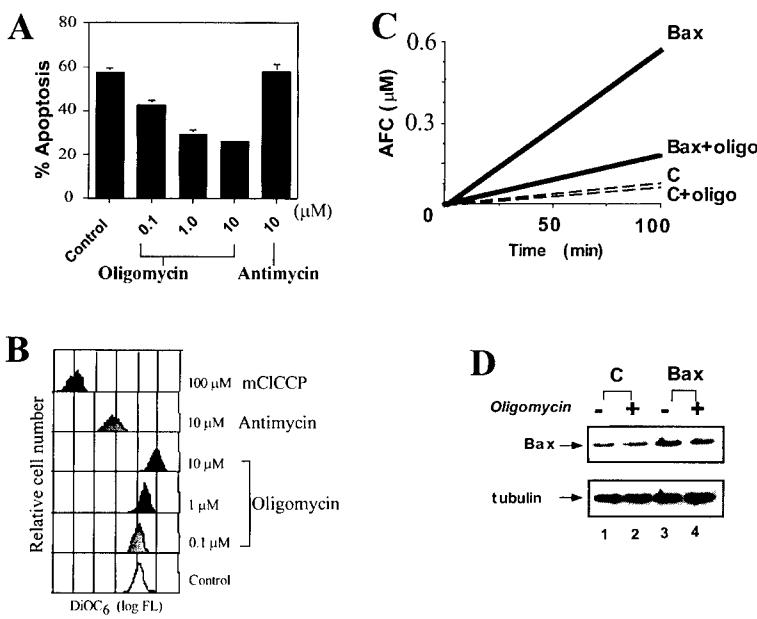


Figure 5. The  $F_0F_1$ -ATPase Inhibitor Oligomycin Suppresses Bax-Induced Apoptosis and Caspase Activation in 293T Cells

(A) 293T cells were cultured in DMEM-high glucose medium to maintain ATP supplies by glycolysis. Four hours after transfection with 9 μg of pcDNA-Bax (Bax) or control pcDNA (C) plasmids with 1 μg of pEGFP, the culture medium was exchanged with fresh medium containing or lacking 0, 0.1, 1, or 10 μM oligomycin or 10 μM antimycin A. After an additional 8 hr of culture, the cells were collected. In (A), the percentage of GFP<sup>+</sup> cells with apoptotic morphology was determined by DAPI-staining (mean  $\pm$  SD; n = 3).

(B) 293T cells were recovered from cultures of untransfected cells and incubated with DiOC<sub>6</sub>, followed by FACS analysis. Data represent log fluorescence versus relative cell number. As a control for specificity of DiOC<sub>6</sub> labeling, an aliquot of the control untreated cells was exposed to the protonophore mCICCP for 15 min prior to incubation with DiOC<sub>6</sub>.

(C) Lysates derived from cells that had been cultured with or without 10 μM of oligomycin were prepared and normalized for total protein content, and caspase activity was measured based on hydrolysis of DEVD-AFC (Deveraux et al., 1997). Typical substrate hydrolysis progress curves are shown (representative of three experiments).

(D) Aliquots of the same lysates employed for caspase assays were subjected to immunoblot analysis, employing anti-hu Bax antiserum with ECL-based detection. The blot was subsequently reprobed with anti-tubulin antibody to verify loading of equivalent amounts of total protein.

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to pharmacological studies employing oligomycin. In mammalian cells, unlike in yeast, oligomycin is toxic and leads secondarily to mitochondrial PTP opening and either apoptosis or necrosis, depending on the particular cells and circumstances evaluated (Castedo et al., 1996). Cell death caused by oligomycin, however, can be delayed by culturing in high glucose-containing medium, which helps to maintain ATP levels via glycolysis (Eguchi et al., 1997; Leist et al., 1997). We therefore explored the effects of oligomycin in human 293T kidney epithelial cells grown in high glucose medium, using a transient Bax transfection assay to induce apoptosis and activation of cell death proteases (caspases) (Zha et al., 1996; Deveraux et al., 1997; Jürgensmeier et al., 1997). For all experiments, oligomycin was added 4 hr after transfections; caspase activity and apoptosis were then measured after an additional 8 hr of culture. Thus, the experiments were performed within the first 12 hr after Bax transfections, before oligomycin caused cell death (>90% trypan blue dye exclusion in control cultures).

As shown in Figure 5A, oligomycin reduced the percentage of apoptotic cells in cultures of Bax-transfected 293T cells in a dose-dependent manner, with concentrations of 1–10 μM oligomycin preventing approximately half of the Bax-induced cell death. DiOC<sub>6</sub>-based measurement of mitochondrial membrane potential demonstrated that these concentrations of oligomycin resulted in hyperpolarization of mitochondria in 293T cells, consistent with a block of the proton pump causing accumulation of H<sup>+</sup> ions in the intermembrane space of these organelles (Figure 5B). Acute exposure of the cells to the protonophore mCICCP confirmed that the DiOC<sub>6</sub> staining was dependent on the mitochondrial pH gradient (Figure 5B), thus verifying the specificity of this assay.

In contrast to oligomycin, culturing Bax-transfected

293T cells with the respiratory complex III inhibitor antimycin A did not impair Bax-induced apoptosis under these conditions, but did markedly reduce mitochondrial  $\Delta\Psi$  (Figure 5B). These findings in mammalian cells thus support the observations obtained with yeast, demonstrating again that respiration is unnecessary for Bax-mediated cell death.

Since gene transfer-mediated overexpression of Bax has been shown to induce activation of caspases that can cleave the substrate peptide DEVD (Deveraux et al., 1997; Jürgensmeier et al., 1997), we measured the effects of oligomycin treatment on Bax-induced activation of DEVD-cleaving caspases using lysates from the transfected 293T cells. As shown in Figure 5C, 293T cells transfected with pcDNA3-Bax contained markedly elevated levels of caspase activity compared to control transfected cells. Addition of 1–10 μM oligomycin to the cultures substantially reduced the amount of Bax-induced caspase activity (Figure 5C and data not shown).

Under these same conditions, ATP levels were maintained to within ~95% of control levels for 293T cells treated with 1 μM oligomycin (32  $\pm$  3 nmol/mg protein versus 34  $\pm$  3 nmol/mg protein) and to within ~75% of control levels for cells treated with 10 μM oligomycin (25  $\pm$  2 nmol/mg protein). Thus, the oligomycin-mediated protection against Bax-induced apoptosis cannot be ascribed to reduced ATP levels. Oligomycin also did not impair production of the expected 21 kDa Bax protein in 293T cells (Figure 5D).

When used at high concentrations in vitro, oligomycin has been reported to inhibit the plasma membrane Na-K ATPase ( $IC_{50} \sim 5 \mu M$ ) (Decottignies et al., 1995). We therefore tested the effect of the Na-K ATPase inhibitor ouabain on Bax-induced apoptosis in 293T cells, but found that even at 100 μM, ouabain had no influence on Bax function (not shown). Based on the above results,

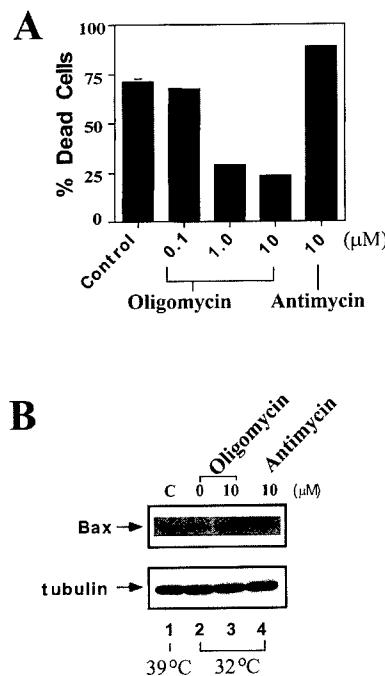


Figure 6. Oligomycin Inhibits p53-Induced Apoptosis

BRK cells that contain ts-p53 (Han et al., 1996) were maintained at a nonpermissive temperature of 39°C, then cultured at 32°C to induce p53 in the presence or absence of 0.1–10 μM oligomycin or 10 μM antimycin A. In (A), the percentage of dead cells was determined by DAPI staining 12 hr after shifting to 32°C (mean  $\pm$  SD;  $n = 3$ ). In (B), Lysates were prepared from BRK cells that had been cultured at 39°C (lane 1) as a control (C) or at 32°C (lanes 2–4) without or with 10 μM oligomycin or 10 μM antimycin for 12 hr, normalized for total protein content (5 μg/lane), and analyzed by immunoblotting using anti-Bax antiserum with ECL-based detection. The same blot was reprobed with anti-tubulin.

therefore, we conclude that the  $F_0F_1$ -ATPase proton pump is either required for optimal function of Bax in 293T cells or enhances Bax's ability to induce apoptosis in these human cells.

#### Oligomycin Inhibits p53-Induced Apoptosis

The induction of apoptosis in baby rat kidney (BRK) cells by p53 has been shown to be Bax-dependent (Han et al., 1996; Sabbatini et al., 1997). We therefore employed BRK cells that express a temperature-sensitive mutant of p53, and examined the impact of culturing these cells with various concentrations of oligomycin at either the permissive temperature of 32°C where p53 is active and apoptosis ensues or at the nonpermissive temperature of 37–39°C where p53 is inactive. As in the prior experiments, these cells were grown in high glucose medium to maintain ATP levels through glycolysis.

Oligomycin reduced the percentage of apoptotic cells by approximately two-thirds when ts-p53 BRK cells were cultured at 32°C to active p53 (Figure 6A). Under these same conditions, ATP levels were maintained at  $\sim$ 100% of control levels in BRK cells treated with 1 μM oligomycin and to within  $\sim$ 70% of control for 10 μM oligomycin (not shown). In contrast, antimycin A had no apparent effect on p53-induced apoptosis in BRK cells (Figure 6A), yet reduced ATP levels more than oligomycin. Immunoblot analysis of BRK cells demonstrated

that neither oligomycin nor antimycin A (10 μM) impaired p53-induced expression of Bax when the cells were cultured at the permissive temperature of 32°C (Figure 6B). Taken together, therefore, these data provide further evidence that Bax-dependent apoptosis requires the mitochondrial  $F_0F_1$ -ATPase proton pump.

#### Discussion

Here we present genetic evidence that the mitochondrial  $F_0F_1$ -ATPase proton pump is required for Bax-induced cell death in yeast. By employing oligomycin, a specific inhibitor of the proton pump, we also found that mitochondrial  $F_0F_1$ -ATPase is apparently required for at least optimal induction of apoptosis and activation of caspases by Bax in mammalian cells. Based on currently available information, Bax and the  $F_0F_1$ -ATPase proton pump are thought to reside in different mitochondrial membranes, with Bax associated with the outer membranes oriented primarily toward the cytosol and the  $F_0F_1$ -ATPase in the inner membrane (Figure 7). Presumably, therefore, these proteins do not physically interact, though the tendency of Bcl-2 family proteins to concentrate at the junctional complexes of mitochondria, where the inner and outer membranes come into contact (Krajewski et al., 1993; de Jong et al., 1994), may create opportunities for a direct interaction. This might be particularly true when Bax is integrated into membranes within its capacity as a channel-forming protein, which is speculated to involve the insertion of the predicted 5th and 6th  $\alpha$  helices of Bax through the lipid bilayer (reviewed in Reed, 1997).

Barring a direct physical interaction, how then might the  $F_0F_1$ -ATPase contribute to Bax-induced cell death? At least two potential explanations can be entertained. First, loss of the proton pump might prevent Bax from integrating into the outer membrane to form a channel. In this regard, the channels formed in vitro by Bax, as well as by Bcl-2 and Bcl-X<sub>L</sub>, are voltage-dependent and their activities are modulated by pH (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997). For example, the conductances of Bax channels in planar bilayers are reportedly higher at neutral than at acidic pH, whereas Bcl-2 channels open more frequently at lower pH. In addition, most Bcl-2 family proteins including Bax contain a glutamic acid residue between the  $\alpha$ 5 and  $\alpha$ 6 helices. Protonation of this residue at lower pHs therefore could destabilize the membrane-inserted channel-forming conformation, allowing these two helices to more easily slip back out of the planar bilayer. Thus, alterations of the voltage potential across the inner mitochondrial membrane where the  $F_0F_1$ -ATPase resides or changes of pH at the surface of the inner membrane theoretically could interfere with Bax channel activity, assuming Bax can be influenced by the local voltage potential and pH gradient, particularly at the junctional complexes. The insertion of the  $\alpha$ 5 and  $\alpha$ 6 helices of Bax into membranes could also provide a way for exposing the BH3 domain of Bax so that the hydrophobic face of this  $\alpha$  helix is available for dimerization with Bcl-2 family proteins (Figure 7), as revealed by recent structural studies (Sattler et al., 1997). BH3 domain-mediated dimerization could play an important role in

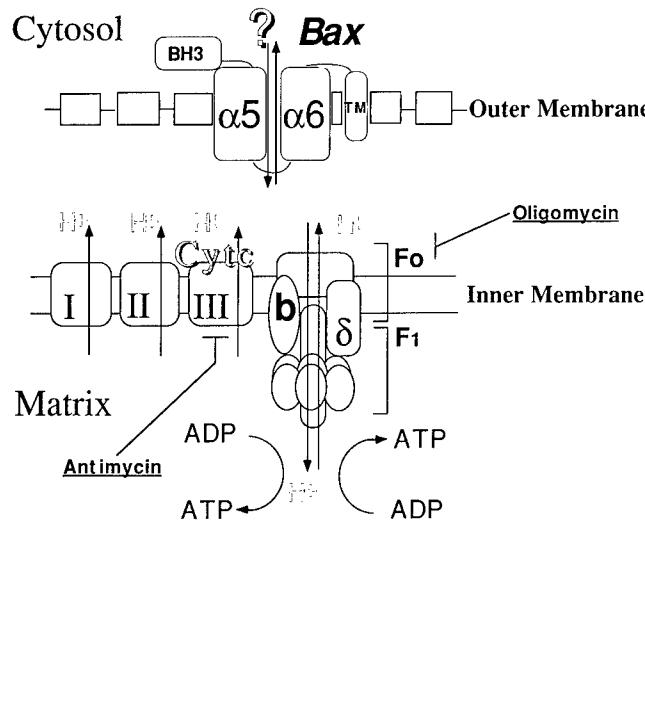


Figure 7. Schematic of Potential Relations between Mitochondrial  $F_0F_1$ -ATPase Proton Pump and Bax

The diagram depicts mitochondria, showing the inner and outer membranes. The outer membrane is thought to be porous because of porin. Bax in the outer membrane is anchored via a C-terminal transmembrane (TM) domain. During channel formation, the predicted 5th and 6th  $\alpha$  helices of Bax are speculated to penetrate the lipid bilayer. The BH3 domain ( $\alpha$ 2 helix) is located on the cytosolic side of the membrane and can mediate dimerization with Bcl-2 and related proteins. The  $F_0F_1$ -ATPase proton pump resides in the inner membrane. The  $\beta$  (equivalent to subunit 4 in yeast) and  $\delta$  subunits are indicated. The  $\beta$  and  $\delta$  subunits of  $F_0$  and  $F_1$ , respectively, play important roles in connecting the  $F_0$  proton channel and  $F_1$  ATPase portions together. The transport of  $H^+$  ions by the proton pump is reversible, and can either consume or generate ATP. Oligomycin shuts off the pump, such that protons cannot be transported in either direction.

The respiratory chain complexes I, II, and III extrude protons into the intermembrane space. Complex III is inhibited by antimycin A and associated with cytochrome c.

modulating interactions of Bcl-2 and Bcl-X<sub>L</sub> with CED-4 or other proteins (Reed, 1997), irrespective of ion-channel formation, at least in mammalian cells.

A second possible explanation for why the  $F_0F_1$ -ATPase is required for Bax-mediated lethality in yeast is that the proton pump may be a downstream effector of Bax. In this regard, the  $F_0F_1$ -ATPase can operate in both forward and reverse directions, either transporting protons into the matrix down their concentration gradient and creating ATP, or pumping protons out of the matrix while consuming ATP (Figure 7). It is conceivable, therefore, that Bax channels render the outer membrane more porous, causing a faster dissipation of the proton gradient than usual through leakage of  $H^+$  ions into the cytosol. As a secondary consequence, the  $F_0F_1$ -ATPase proton pump would be predicted to run in reverse, thus consuming ATP and alkalinizing the matrix by extruding protons. Since alkalinization of the matrix has been shown to cause opening of the mitochondria permeability transition (PT) pore in both mammalian and yeast mitochondria (Bernardi et al., 1994; Ichas et al., 1997; Jung et al., 1997), the  $F_0F_1$ -ATPase could theoretically facilitate Bax-induced cell death by this mechanism. In this regard, yeast mitochondria have been reported recently to have evidence of a PT pore that can be induced to open by matrix alkalinization (Jung et al., 1997).

In previous studies where oligomycin was used under conditions designed to maintain ATP levels and prevent necrosis, it was observed that cell death induced by etoposide and dexamethasone was inhibited (Eguchi et al., 1997; Leist et al., 1997). In contrast, apoptosis induced by anti-Fas antibody is apparently not dependent on the  $F_0F_1$ -ATPase, since Fas can still induce apoptosis in cells exposed to oligomycin in high-glucose media. Though this observation clearly suggests that the  $F_0F_1$ -ATPase is not absolutely necessary for apoptosis, it

does not discount the possibility that the proton pump contributes to Bax-induced cell killing since Fas-induced apoptosis appears to be relatively Bax-independent (reviewed in Vaux and Strasser, 1996) whereas apoptosis induced by etoposide and dexamethasone can be assisted by Bax (Brady et al., 1996).

In summary, the data reported here demonstrate a role for the mitochondrial  $F_0F_1$ -ATPase in Bax-induced cell death. Though other explanations are possible, we suspect that the functional interaction between Bax and the proton pump is a manifestation of Bax's ability to function as a channel protein in mitochondrial membranes. However, Bax can promote cell death in mammalian cells by at least two mechanisms: (a) by forming channels in membranes; and (b) by dimerizing with anti-apoptotic Bcl-2 family proteins and thereby interfering with their actions independent of channel formation (Reed, 1997). In yeast, we hypothesize that the bioactivity of Bax is entirely dependent on its ability to form channels, since these simple unicellular organisms appear to lack Bcl-2 and CED-4 homologs. In mammalian cells, however, both mechanisms are likely to be operative. It remains to be determined which of these two mechanisms for Bax-mediated cell death (channel formation versus Bcl-2 antagonist) is quantitatively more important in mammalian cells, but cellular context and the specific cell death stimulus involved are likely to be highly important. Regardless, the data presented here suggest that applications of yeast genetics may provide novel insights into the channel-dependent mechanisms of Bax-induced cell death.

#### Experimental Procedures

##### Plasmids

YEp51-Bax, pEG202-Bax, and pcDNA-Bax have been described (Zha et al., 1996). The Bax cDNA from pEG202-Bax was subcloned

into the EcoRI and Xhol sites of pGilda (gift of C. Kaiser [MIT]). The YCp50 plasmid (ATCC37419) and yeast genomic library (ATCC37415) were obtained from American Type Culture Collection (Rockville, Maryland).

#### Yeast Methods

Yeast strains and plasmids used for these studies have been described previously (Velours et al., 1989; Paul et al., 1992; Giraud and Velours, 1994; Zha et al., 1996). For generation of Bax-resistant mutant yeast, EGY48 strain was mutagenized with MMNG (Sigma, Inc.) using routine methods (Guthrie and Fink, 1991). After treatment with MMNG for 10 min, yeast cells were grown for 4 hr in YPD media and then transformed by a LiOAc method with the *GAL1* promoter-containing plasmid YE<sub>p</sub>51-Bax and plated on minimal medium supplemented with required amino acid (MM-A) containing 1% raffinose and 2% galactose. Surviving clones were picked from plates and grown in MM-A with glucose prior to transformation with the *ADH1* promoter-containing plasmid pEG202-Bax (Sato et al., 1994) and selection on MM-A/2% glucose plates. Resistant mutants were mated with Myy290 strain (*Mata*, *his3*, *ura3*, *leu2*), and the resulting diploid cells were subjected to tetrad analysis (Guthrie and Fink, 1991). BRM1 cells displayed precisely 50% inheritance of the Bax-resistant phenotype.

#### Complementation Cloning

BRM1 cells were transformed with the *GAL1* promoter-containing plasmid pGilda-Bax and grown in MM-A with glucose to a density of  $2-4 \times 10^7$  cells/ml. These cells were then transformed using a LiOAc method with 2  $\mu$ g of a yeast genomic library in YCp50 (ATCC) and 20  $\mu$ g of salmon sperm DNA (transformation efficiency  $1 \times 10^4$  to  $2 \times 10^4$  per  $\mu$ g DNA). Transformed cells were first plated on MM-A with glucose and then replica-plated to MM-A with galactose. From  $\sim 2 \times 10^4$  independent colonies tested, 8 clones were identified that did not grow on MM-A/galactose plates. Plasmids were recovered from these 8 candidates, and BRM1 cells were retransformed with these library plasmid together with pGilda-Bax, thus confirming restoration of sensitivity to Bax to approximately the same level as wild-type yeast for 1 of them. The ends of this plasmid were sequenced by primers flanking the cloning site in YCp50 (5'-CGATCATGGCGACCA CACCCGTCCT-3' and 5'-GGTGATGCCGCCACGATGCGTCCT-3'). The DNA sequence results were compared with the Yeast Genomic Data Base using dbFAST (Stanford University).

#### Yeast Cell Viability Assays

Single colonies of yeast cells transformed with pGilda or pGilda-Bax were grown in 10–20 ml of MM-A/glucose with vigorous aeration at 30°C to an optical density of 0.4–0.5 OD<sub>600</sub> nm. Cells were pelleted by centrifugation (1000  $\times$  g) for 10 min and washed three times in MM-A/galactose before resuspending in 20 ml of MM-A/galactose and culturing half with 10  $\mu$ M oligomycin and half with ethanol solvent control (final 0.1%). Oligomycin or ethanol was added to MM-A/galactose medium every 12 hr to ensure maintenance of adequate levels of drug. After culturing for various times at 30°C, a 0.5 ml aliquot of cells was removed for trypan blue dye exclusion assay, counting at least 300 total (live and dead) cells. Alternatively, the total cell density of cultures was determined, and either 1000 or 3000 cells were spread on MM-A/glucose plates, followed by culturing at 30°C for 4 days. The number of colonies on plates from the 0 hr cultures was designated as 100%.

#### Mammalian Cell Transfections and Apoptosis Assays

293T cells were cultured for 12 hr in DMEM-high glucose (4500 mg glucose/l) medium supplemented with 10% fetal bovine serum (FBS) at a density of  $10^6$  cells in 3.6 ml of medium per 6 cm diameter dish or  $3 \times 10^6$  cells in 10 ml per 10 cm dish. Fresh medium was exchanged and 4 hr later the cells were transfected with 10  $\mu$ g of pcDNA3-Bax versus parental pcDNA3 plasmid (10 cm dishes) or were cotransfected with 1  $\mu$ g of pEGFP (Clontech Laboratories, Inc.) and either 9  $\mu$ g of pcDNA3-Bax or pcDNA3 control plasmid (6 cm dishes). Four hours after the transfection, the medium was changed with fresh media containing 10  $\mu$ M oligomycin, 10  $\mu$ M antimycin A, or 0.1% ethanol (solvent). After culturing for an additional 8 hr, both the floating and attached cells were harvested. Half

of the recovered cells were used for immunoblot assays and the remainder were used for either caspase activity assays (Deveraux et al., 1997) or for DAPI staining (Zha et al., 1996).

BRK cells expressing ts-p53 (Subramanian et al., 1995) were maintained in DMEM-high glucose 10% serum medium at the nonpermissive temperature of 39°C and cultured at either  $5 \times 10^4$  cells/0.1 ml in 96-well flat bottom plates or at  $10^6$  cells/5 ml in 6 cm dishes for cell death assays. The medium was then changed with fresh 32°C medium, and cells were cultured at 32°C with or without various concentrations of oligomycin or antimycin A for 12 hr. The percentage of viable cells was determined by trypan blue dye exclusion, or cell lysates were prepared for immunoblot analysis of Bax expression.

#### Immunoblot Assays

Whole cell lysates were normalized for total protein content, and immunoblot assays were performed as described previously using 0.1% (v/v) anti-LexA rabbit serum (Zha et al., 1996) or either anti-human Bax or anti-mouse/rat Bax rabbit sera (Krajewski et al., 1994; Krajewski et al., 1995).

#### ATP Measurements

Cellular ATP content was measured as previously reported (Kane et al., 1985) using firefly lantern extract (Luciferase-luciferin, Sigma) with a luminometer. Data were normalized relative to total protein content of cell lysates.

#### Measurements of Mitochondrial $\Delta\Psi$

Mitochondrial  $\Delta\Psi$  was measured using DiOC<sub>6</sub> (Molecular Probes, Inc.) as described (Castedo et al., 1996).

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# Bax Inhibitor-1, a Mammalian Apoptosis Suppressor Identified by Functional Screening in Yeast

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## Summary

The mammalian proapoptotic protein Bax confers a lethal phenotype when expressed in yeast. By exploiting this phenotype, we have identified a novel human Bax inhibitor, BI-1. BI-1 is an evolutionarily conserved integral membrane protein containing multiple membrane-spanning segments and is predominantly localized to intracellular membranes, similar to Bcl-2 family proteins. Moreover, BI-1 can interact with Bcl-2 and Bcl-X<sub>L</sub> but not Bax or Bak, as demonstrated by *in vivo* cross-linking and coimmunoprecipitation studies. When overexpressed in mammalian cells, BI-1 suppressed apoptosis induced by Bax, etoposide, staurosporine, and growth factor deprivation, but not by Fas (CD95). Conversely, BI-1 antisense induced apoptosis. BI-1 thus represents a new type of regulator of cell death pathways controlled by Bcl-2 and Bax.

## Introduction

Bcl-2 family proteins are centrally involved in control of programmed cell death (PCD), with some inhibiting (Bcl-2 and Bcl-X<sub>L</sub>) and others promoting (Bax and Bak) apoptosis (reviewed in Reed, 1994; Yang and Korsmeyer, 1996; Kroemer, 1997). The ability of Bcl-2 family proteins to regulate cell life and death is conserved across evolution. For example, the nematode *Caenorhabditis elegans* contains a Bcl-2 homolog, CED-9, that is essential for the viability of these animals, and expression of the human Bcl-2 protein in *C. elegans* can rescue CED-9-deficient worms (Vaux et al., 1992; Hengartner and Horvitz, 1994). The human Bcl-2 protein can also block apoptotic cell death in insect cells (Alnemri et al., 1992), and human Bcl-2 can protect some mutant yeast strains from death induced by oxidative injury (Kane et al., 1993).

The biochemical mechanism of action of Bcl-2 and its homologs is controversial (reviewed by Reed, 1997a). Recent determination of the three-dimensional structure of Bcl-X<sub>L</sub> suggests similarity to the pore-forming domains of some bacterial toxins, particularly diphtheria toxin and the colicins (Muchmore et al., 1996). These toxins function by forming channels in membranes that transport either ions or proteins (Donovan et al., 1981; Cramer et al., 1995). Several members of the Bcl-2 family, including Bcl-2, Bcl-X<sub>L</sub>, and Bax, are capable of forming ion-conducting channels in synthetic membranes *in vitro* (Antonsson et al., 1997; Minn et al., 1997; Schendel et al., 1997; Schlesinger et al., 1997). Thus, some Bcl-2 family proteins may have intrinsic activities as channel

proteins, irrespective of other functions related to their ability to interact with several types of proteins in cells (Reed, 1997a).

The mammalian Bax protein confers a lethal phenotype when expressed in either the budding yeast *Saccharomyces cerevisiae* or the fission yeast *Schizosaccharomyces pombe* (Sato et al., 1994; Greenhalf et al., 1996; Zha et al., 1996; Ink et al., 1997; Jürgensmeier et al., 1997). The cell death induced by Bax or its close relative Bak is not attributable to nonspecific toxicity caused by overexpression of a heterologous protein, since antiapoptotic Bcl-2 proteins can rescue yeast from Bax/Bak-induced lethality. Moreover, mutants of Bcl-2 and Bcl-X<sub>L</sub> that fail to protect in mammalian cells are inactive at suppressing Bax-induced cell death in yeast. In addition, certain mutations of Bax and Bak that abolish their proapoptotic function in mammalian cells also abrogate their lethal effects in yeast (Zha et al., 1996; Ink et al., 1997). Recently, Bax has been shown to induce release of cytochrome c from mitochondria in yeast (Mannon et al., 1997), which further suggests commonalities in the mechanisms by which Bax induces death in yeast and mammalian cells, given that apoptosis is often associated with release of cytochrome c from mitochondria in mammalian cells (reviewed by Reed, 1997b). In addition, a requirement for the F<sub>0</sub>F<sub>1</sub>-ATPase proton pump of mitochondria has been demonstrated for optimal cell death-inducing function of Bax in both yeast and mammalian cells (Matsuyama et al., 1998 [this issue of *Molecular Cell*]).

By exploiting the lethal phenotype of Bax in yeast, we reasoned that it should be possible to identify mammalian proteins that suppress Bax function by screening cDNA expression libraries for clones that rescue yeast from Bax-mediated cell death.

## Results

### cDNA Cloning of Human BI-1

A yeast strain (QX95001) was constructed by transforming the Bax-expression plasmid YEp51-Bax, encoding the full-length mouse Bax protein under the control of the galactose-inducible yeast *GAL10* promoter (Zha et al., 1996), into strain BF264-15Dau (Lew et al., 1991). These yeast cells died upon transfer from glucose to galactose-containing medium, which induces the *GAL10* promoter in this plasmid and leads to accumulation of Bax protein in yeast (Zha et al., 1996). A human HepG2 cDNA expression library (Lew et al., 1991) was transformed into the QX95001 strain and screened for Bax-resistant transformants by plating on galactose-containing solid medium. From a screen of  $\sim 10^6$  transformants, 17 displayed Bax resistance. Of these, 4 were determined to be dependent on the introduced cDNA library plasmid by "con-committant-loss" assay (Ausubel et al., 1991). The nucleotide sequences of 3 of these cDNA clones encoded the same protein, designated

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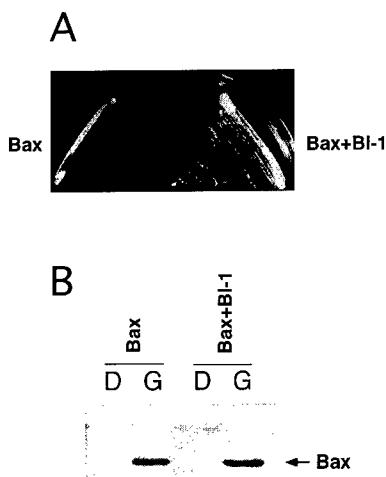


Figure 1. BI-1 Suppresses Bax-Induced Yeast Cell Death

Either control vector or BI-1-encoding expression plasmid (isolated from the HepG2 library) was transformed into cells of yeast strain QX95001 (harboring YEp51-Bax).

(A) Transformants were streaked on galactose-containing synthetic medium lacking uracil and leucine. Photograph was taken after a 4 day incubation at 30°C.

(B) Protein extracts were prepared from QX95001 transformants used in (A) that contained control or BI-1-encoding plasmids. Cells were grown in glucose-containing medium (D = dextrose) and then transferred to galactose-containing medium (G) for 20 hr. Total protein extracts (20 µg/lane) were subjected to SDS-PAGE and immunoblot analysis using anti-mBax antiserum.

BI-1, for Bax Inhibitor-1. BI-1 did not interfere with production of the Bax protein in yeast, as determined by immunoblot analysis (Figure 1).

#### The Predicted BI-1 Protein Contains Several Membrane-Spanning Segments

All three BI-1 cDNAs obtained by the functional yeast screen contained an open reading frame (ORF) encoding a predicted protein of 237 amino acids. The predicted AUG start codon for this ORF was within a favorable context for translation initiation (Kozak, 1997) and was preceded by an in-frame stop codon. A search against the available nucleotide sequence databases using the BLAST program (Altschul et al., 1990) revealed that BI-1 is essentially identical to TEGT ("testis enhanced gene transcript"), a cDNA previously cloned fortuitously during an attempt to identify testis-specific genes (Walter et al., 1995). The rat TEGT protein has also been described and shares 90% identity (95% similarity) with the human protein (Walter et al., 1994). Additional previously undescribed homologs of BI-1 were also identified by BLAST searches in mouse, and possibly in the nematode *C. elegans* and the plant *Arabidopsis thaliana*. The mouse BI-1 protein, as deduced from ESTs #AA015124, AA275830, AA467259, AA107704, and W59401 is 237 amino acids in length and shares 92% identity (95% similarity) with the human BI-1 protein (Figure 2). The *C. elegans* ORF (EMBL #Q20241) encodes a protein of 241 amino acids that shares 21% overall amino acid identity (37% similarity) with human BI-1. This percentage homology shared by the nematode and mammalian BI-1 is similar to that reported for Ced-9 and Bcl-2,

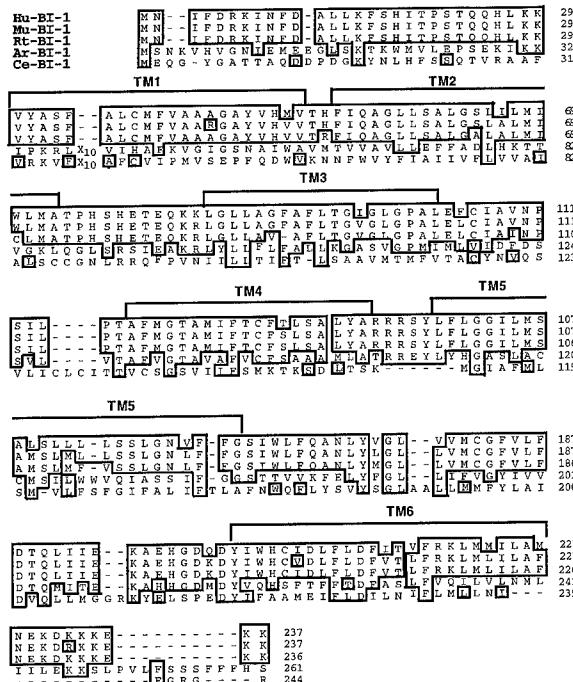


Figure 2. BI-1 Is an Evolutionarily Conserved Protein with Several Predicted Membrane-Spanning Domains

The predicted amino acid sequences of human, rat, and mouse BI-1-proteins, as well as the homologous *C. elegans* and *Arabidopsis* proteins are aligned, with identical residues in boxes. The predicted 6 TM domains of the human BI-1 protein are indicated in brackets.

which share only 23% identity in their amino acid sequences. The putative *Arabidopsis* homolog (EMBL #Z97343) is 261 residues in length, sharing 29% amino acid sequence identity (45% similarity) with the human BI-1 protein.

Based on Kyte-Doolittle hydropathy analysis (Kyte and Doolittle, 1982), the BI-1 protein is predicted to contain 6 or 7 transmembrane (TM) domains. The C termini of the mammalian BI-1 proteins are comprised of basic amino acids resembling some nuclear targeting sequences (Dingwall and Laskey, 1991), but otherwise the predicted proteins lack motifs that suggest a function. Of note, BI-1 does not contain any of the conserved BH domains of Bcl-2 family proteins. By searching a transmembrane protein database (TMbase) using the TMpred program (Hofmann and Stoffel, 1993), the most favored membrane topology for mammalian BI-1 is with 6 membrane-spanning domains and both the N and C termini oriented toward the cytosol. The putative transmembrane segments are predicted to assume mostly an  $\alpha$ -helical conformation. Triton X-114 partitioning studies confirmed that BI-1 is an integral membrane protein (data not presented). RNA blot analysis indicated that BI-1 is widely expressed *in vivo*, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (not shown).

#### BI-1 Inhibits Bax-Induced Apoptosis in Mammalian Cells

Transient transfection of Bax-encoding expression plasmids induces apoptosis in the human embryonic kidney

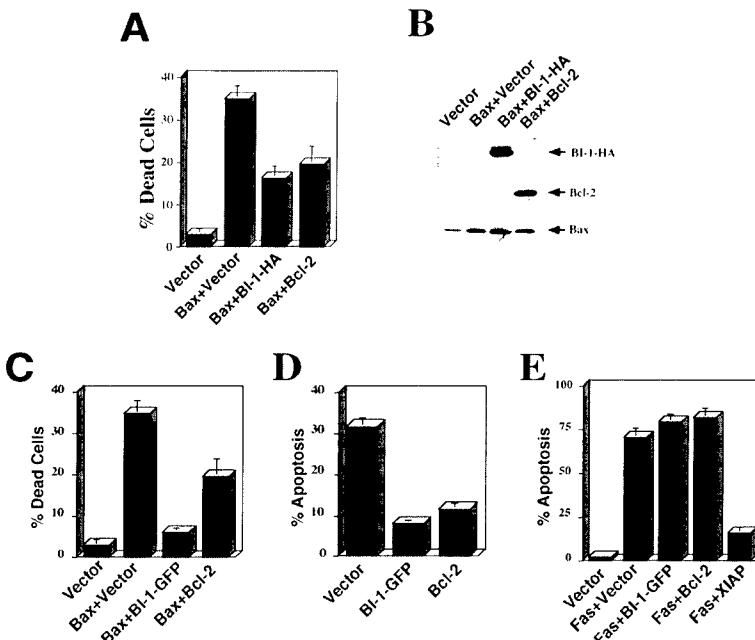


Figure 3. BI-1 Inhibits Cell Death Induced by Bax or Serum Withdrawal but Not Fas

(A) BI-1 protects against Bax-induced apoptosis in 293 cells. 293 cells were transfected with either 9  $\mu$ g of vector control, or cotransfected with 3  $\mu$ g of Bax plasmid and 6  $\mu$ g of either control vector (pcDNA3) or plasmids encoding BI-1 (with a C-terminal HA tag) or Bcl-2. One day after transfection, floating and adherent cells (after trypsinization) were pooled. A portion of the pooled cells was subjected to vital dye trypan blue exclusion assay counting at least 300 cells (mean  $\pm$  SD;  $n = 3$ ).

(B) Extracts were prepared from another portion of the transiently transfected 293 cells described in (A) above and subjected to SDS-PAGE/immunoblot analysis. The blot was sequentially probed with anti-HA monoclonal antibody, anti-hBax antiserum, and anti-Bcl-2 antiserum, with stripping between each detection. The predicted band corresponding to the  $\approx$ 30 KD BI-1-HA protein is indicated with an arrow.

(C) 293 cells were transiently transfected with either vector control (9  $\mu$ g) or cotransfected with 3  $\mu$ g of Bax plasmid and 6  $\mu$ g of either BI-1-GFP or Bcl-2 plasmid. Trypan blue dye

positive cells (%) were determined 1 day later (mean  $\pm$  SD;  $n = 3$ ). The expression of GFP-tagged BI-1 was verified by fluorescence microscopy and by immunoblotting using an anti-GFP monoclonal antibody (now shown).

(D) GM701 cells were cotransfected with a  $\beta$ -gal reporter plasmid (0.5  $\mu$ g) and the indicated plasmids (4.5  $\mu$ g each). 18 hr after transfection, cells were washed and cultured in DMEM containing 0.1% FBS for another 30 hr. Floating and adherent cells were fixed and stained with X-gal. The percentage of blue cells (transfected) with apoptotic morphology was determined (mean  $\pm$  SD;  $n = 3$ ).

(E) 293 cells were transfected with either vector control (8.5  $\mu$ g) or cotransfected with 0.5  $\mu$ g of Fas-encoding plasmid and 8  $\mu$ g of either vector, or BI-1-GFP-, or Bcl-2- or XIAP-encoding plasmids. GFP-encoding plasmid (0.5  $\mu$ g) was included in all transfections. 20 hr after transfection, both floating and adherent cells (after trypsinization) were pooled, fixed, and stained with DAPI (Zha et al., 1996). The percentage of GFP-positive cells with fragmented or condensed nuclei (apoptotic) was determined (mean  $\pm$  SD;  $n = 3$ ).

cell line 293 (Zha et al., 1996). Consequently, pcDNA3-hBax was cotransfected with equal amounts of pcDNA3 parental vector (used as a negative control), pcDNA3-BI-1-HA (HA-tagged BI-1), or pRc/CMV-Bcl-2 (used as a positive control). One day later, both the floating and adherent cells were collected and subjected to the trypan-blue vital dye exclusion assay. BI-1 suppressed Bax-induced cell death in 293 cells to a similar extent as Bcl-2 (Figure 3A). DAPI staining of 293 cell nuclei confirmed that Bax-induced cell death occurred by apoptosis (not shown). Immunoblot analysis demonstrated that BI-1 does not interfere with Bax production in 293 cells (Figure 3B).

#### BI-1 Inhibits Apoptosis Induced by Growth Factor Deprivation, Etoposide, and Staurosporine, but Not Fas

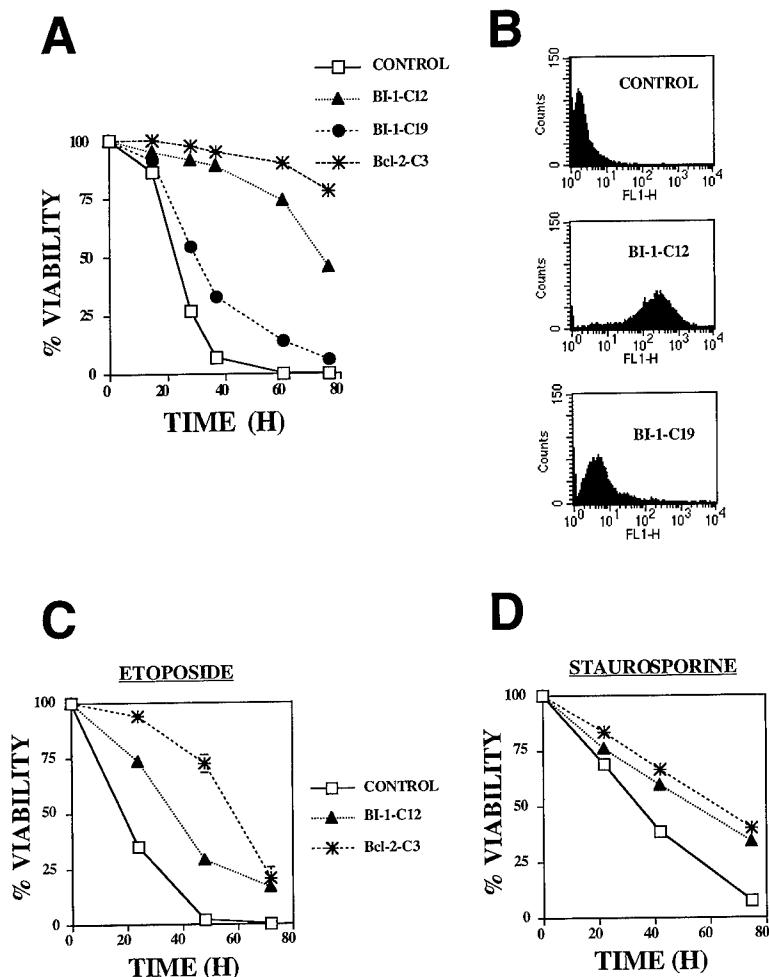
The effects of BI-1 overexpression on apoptosis induced by a variety of stimuli were explored. For many of these experiments, we employed a green fluorescent protein (GFP)-tagged BI-1, because it provided a convenient marker for transfected cells and because BI-1-GFP accumulated to higher levels than the BI-1-HA protein (not shown). Transient transfection studies in 293 cells confirmed that the BI-1-GFP protein retained biological activity as a suppressor of Bax-induced cell death (Figure 3C).

In the human diploid fibroblast line GM701,  $\sim$ 30% of the cells transfected with control GFP-marker plasmid

developed morphological characteristics typical of apoptosis when deprived of serum for  $\sim$ 30 hr. In contrast, GM701 cells transfected with plasmids encoding BI-1-GFP or Bcl-2 were substantially more resistant to serum deprivation, with only  $\sim$ 10% of the cells undergoing apoptosis (Figure 3D). Thus, BI-1 is able to suppress apoptosis induced by growth factor withdrawal in GM701 fibroblasts.

In many types of cells, apoptosis induced by the TNF-family receptor Fas (CD95) is poorly abrogated by Bcl-2 (reviewed by Vaux and Strasser, 1996). We therefore compared the effects of Bcl-2 with BI-1 in 293 cells where apoptosis was induced by transfection of Fas. Cotransfection of either BI-1 or Bcl-2 with a Fas-encoding plasmid failed to prevent apoptosis (Figure 3E). In contrast, Fas-induced cell death was markedly suppressed by cotransfection of a plasmid encoding XIAP, a protein that directly binds to and inhibits caspases required for Fas-induced apoptosis (Deveraux et al., 1997).

To further explore the role of BI-1 as an apoptosis inhibitor, we examined its effects in FL5.12, an interleukin-3-dependent pro-B lymphocyte clone previously shown to undergo apoptosis when deprived of IL-3. FL5.12 cells were stably transfected with plasmids encoding BI-1-GFP or Bcl-2 (as a positive control) and subclones obtained that expressed BI-1 or Bcl-2 at high levels. BI-1 transfectants were uniformly more resistant to apoptosis induction by IL-3 deprivation, with the extent of protection correlating roughly with the levels of



BI-1-GFP protein. Figure 4A presents results from two clones, one with only modest and another with high levels of BI-1-GFP protein production. Note that even clones with low levels of BI-1-GFP protein exhibited some resistance to IL-3 deprivation, whereas clones expressing high levels of BI-1-GFP protein displayed pronounced resistance to apoptosis induction by IL-3 withdrawal, with the prolongation in cell survival approaching that seen in Bcl-2-transfected FL5.12 cells. BI-1 overexpression also afforded protection in FL5.12 cells against apoptosis induced by etoposide and staurosporine. Comparisons of FL5.12 cell clones with various levels of BI-GFP expression suggested that BI-1 was generally somewhat less effective than Bcl-2 at conferring resistance against these agents (Figures 4C and 4D, and data not shown).

Taken together, the transfection experiments in mammalian cells (Figures 3 and 4) demonstrate that BI-1 has antiapoptotic activity in epithelial, fibroblastic, and hematopoietic cells, and suggest that BI-1 shares functional similarity with Bcl-2 in delaying cell death induced by Bax, growth factor deprivation, staurosporine, and etoposide, but not Fas.

#### BI-1 Antisense Induces Apoptosis

A BI-1 cDNA was subcloned into pCMV-Neo in reversed orientation and transiently transfected into 293 cells,

Figure 4. BI-1 Protects against Multiple Apoptotic Stimuli in FL5.12 Lymphocytes

(A) BI-1 inhibits IL-3 withdrawal-induced apoptosis in FL5.12 cells. Stably transfected cell lines expressing GFP-tagged BI-1 or untagged Bcl-2 were generated by electroporation. Independent clones were obtained by limiting-dilution for BI-1 (clone 12 and 19) and Bcl-2 (clone 3). Cells were grown to a density of  $\sim 5 \times 10^5$  cells/ml before removing IL-3 from the medium. At various times thereafter, samples were removed and subjected to trypan blue dye exclusion assay (mean  $\pm$  SD;  $n = 3$ ).

(B) Expression of GFP-tagged BI-1 assessed by FACS analysis. Stably transfected FL5.12 cells containing either a negative control plasmid (pcDNA3) (top panel) or plasmid encoding BI-1-GFP were analyzed by FACS. The histograms are presented for clone 12 (middle panel), which expresses BI-1-GFP at high levels, as manifested by a single peak of green fluorescence at  $>2$  logs above the negative control (top panel) and for clone 19 (bottom panel), which expresses BI-1-GFP at levels only  $\sim 0.5$  log above background. (C and D) FL5.12 cells were cultured with IL-3 and either 5  $\mu$ g/ml etoposide (C) or 0.5  $\mu$ M staurosporine. The percentage of viable cells was determined at various times thereafter by trypan blue dye exclusion assay (mean  $\pm$  SD;  $n = 3$ ). Some SD bars are obscured by symbols.

together with a GFP-marker plasmid. The BI-1 antisense (AS) plasmid induced apoptosis of 293 cells in a concentration-dependent manner, whereas control plasmid had no effect (Figure 5A). Lacking antibodies for detection of endogenous BI-1 protein to verify antisense-mediated down-regulation of BI-1 protein, parallel experiments were performed in which 293 cells were cotransfected with plasmids encoding BI-1-Flag protein and the BI-1-AS plasmid. As shown in Figure 5B, the levels of BI-1-Flag protein were markedly decreased in 293 cells that received the BI-1-AS plasmid compared to control transfected cells, as determined by immunoblotting. In contrast, the BI-1-AS plasmid had no effect on the levels of tubulin or other proteins examined, confirming the specificity of the results. These antisense experiments provide further evidence that BI-1 regulates apoptosis in mammalian cells.

#### BI-1 Is Located in Intracellular Membranes Similar to Bcl-2

The Bcl-2 and Bcl-X<sub>L</sub> proteins are associated with intracellular membranes, primarily the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope (Krajewski et al., 1993; González-García et al., 1994), while Bax appears to be localized mostly to mitochondria (Zha et al., 1996). To preliminarily explore the

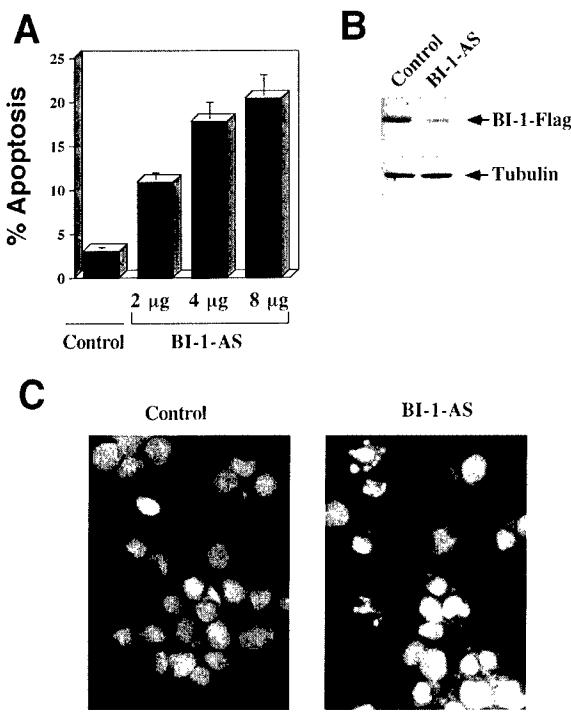


Figure 5. BI-1 Antisense Induces Apoptosis

(A) 293 cells were cotransfected with the indicated plasmids plus 1 μg of GFP-encoding plasmid. The total amount of plasmid used for each transfection was normalized at 9 μg. 30 hr after transfection, both the floating and adherent cells were pooled, fixed, and stained with DAPI. The percentage of apoptosis was determined as the ratio of cells with fragmented or condensed nuclei among GFP-positive cells (mean ± SD; n = 3). Data are representative of three independent experiments.

(B) 293 cells were cotransfected with 1 μg of BI-1-Flag-encoding plasmid in combination with either 8 μg of pCMV-Neo or pCMV-Neo-BI-1-AS. 30 hr after transfection, protein extracts were prepared, normalized for total protein content (30 μg per lane), and subjected to SDS-PAGE/immunoblot analysis using the anti-Flag M2 antibody for detection of BI-1-Flag protein and anti-tubulin antibody to control for loading.

(C) DAPI-stained cells as described in (A) were visualized and photographed under a UV microscope. Note at least four typical apoptotic cells with fragmented or condensed nuclei in the BI-1-AS-transfected population (right panel).

intracellular locations of the BI-1 protein, GFP-BI-1 protein was expressed in several different adherent cell lines (293, Cos-7, GM701). In all three cell lines, fluorescence microscopy demonstrated that BI-1 is exclusively cytosolic and appears to be associated with intracellular membranes in a pattern typical of the endoplasmic reticulum (ER) and its continuity with the nuclear envelope (Figure 6A and data not shown). In contrast, GFP control protein was diffusely distributed throughout the cells. Only a small portion of BI-1 appears to be associated with mitochondrial membranes, based on two-color analysis using a mitochondria-specific fluorescent dye (Figure 6B). Similar results were obtained using a Flag-tagged BI-1 protein instead of GFP-BI-1 (Figure 6Ac).

The intracellular location of BI-1 was also explored by subcellular fractionation experiments. For this purpose, 293T cells were transiently transfected with the BI-1-HA-encoding plasmid or vector control. Cells were lysed

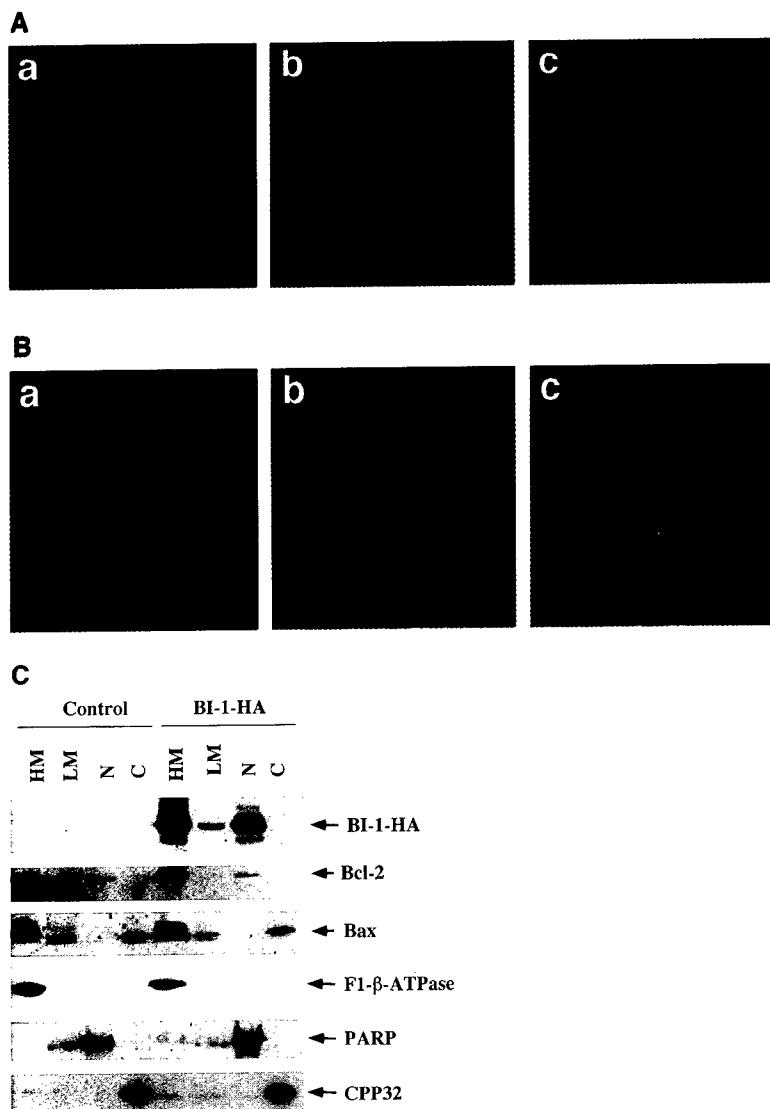
in hypotonic buffer 2 days later and separated into crude subcellular fractions of nuclei (N), heavy membranes (HM), light membranes (LM), and cytosol (C) by differential centrifugation as described (Wang et al., 1996). As shown in Figure 6C, BI-1 was found mostly in the HM and N fractions (inferred as nuclear envelope based on the GFP fusion localization studies), as determined by immunoblot analysis where the fractions were normalized for cell equivalents. A small proportion of the BI-1-HA protein was also found in the LM fraction. The HM fraction contains mitochondria, lysosomes, and rough ER, whereas the LM fraction contains smooth ER, endosomes, and plasma membranes. As a control for the fractionation procedure, the same blot was reprobed with antibodies specific for the mitochondrial inner membrane protein F<sub>1</sub>-β-ATPase, the nuclear protein PARP, and the cytosolic protein CPP32 (caspase-3). Bcl-2 was found essentially in the same subcellular compartments as BI-1, with most of this protein associated with the HM and N fractions (Figure 6C). We conclude therefore that BI-1 is associated with intracellular membranes, based on GFP-tagging experiments, immunofluorescence microscopy, and subcellular fractionation studies.

Recently, it was reported that a subpopulation of Bax molecules in cells are not integrated into membranes, but rather are found within a soluble cytosolic fraction, with the relative proportion of membrane-associated Bax increasing after application of apoptotic stimuli (Hsu et al., 1997). Comparisons of BI-1- and control-transfected 293T cells, however, demonstrated that BI-1 does not substantially change the relative amounts of Bax protein associated with various subcellular compartments (Figure 6C).

#### BI-1 Associates with Bcl-2 In Vivo

The subcellular fractionation data suggest that BI-1 and Bcl-2 colocalize to the same intracellular membranes. To address the question of whether BI-1 and Bcl-2 physically associate in membranes, we performed in vivo cross-linking experiments. Plasmids encoding either Flag-tagged or HA-tagged BI-1 were cotransfected with Bcl-2 into 293 cells. Cells were then incubated 2 days later with the thiol-cleavable chemical cross-linker DTBP. As shown in Figure 7A, both the HA- and Flag-tagged BI-1 proteins could be cross-linked to Bcl-2, suggesting that BI-1 and Bcl-2 come within close proximity to each other in membranes. In contrast, Bax was not cross-linked to BI-1 (data not shown).

To further explore the interaction of BI-1 with Bcl-2 family proteins, we attempted to coimmunoprecipitate BI-1 with Bcl-2, Bcl-X<sub>L</sub>, Bax, and Bak. For these experiments, 293 cells, which contain high levels of Bak but very little endogenous Bcl-2, Bcl-X<sub>L</sub>, or Bax, were transfected with Bcl-2, Bcl-X<sub>L</sub>, or Bax-expression plasmids and either Flag-tagged BI-1 plasmid or empty vector. As shown in Figure 7B, Flag-tagged BI-1 protein specifically coimmunoprecipitated with Bcl-2, and Bcl-X<sub>L</sub> but not Bax or Bak. Testing of several deletion mutants of Bcl-2 revealed that the BH4 domain is required for interactions with BI-1 (not shown). This domain is uniquely found in antiapoptotic but not most proapoptotic members of the Bcl-2 family (Reed, 1997a), presumably explaining



why Bcl-2 and Bcl-X<sub>L</sub> but not Bax and Bak form complexes with BI-1.

#### Discussion

By undertaking a functional screen for Bax suppressors in yeast, we have identified a novel human apoptosis inhibitor, BI-1. BI-1 is highly conserved throughout evolution. It shares no identifiable similarity to Bcl-2 family proteins or any other proteins implicated in PCD. Thus, BI-1 represents a novel type of apoptosis modulator. Interestingly, however, the predicted transmembrane topology and intracellular location of the BI-1 protein are somewhat similar to the presenilins, PS-1 and PS-2, which have been implicated in apoptosis and neurodegenerative disease, though no clear sequence homology is shared between BI-1 and these proteins (Wolozin et al., 1996; Guo et al., 1997).

Because BI-1 was identified by its ability to suppress Bax-induced yeast cell death, in theory, it could function upstream of, at the same level as, or downstream of

#### Figure 6. BI-1 Is Localized to Intracellular Membranes and Cofractionates with Bcl-2

(A) Either the parental pEGFP-N2 vector (a) or plasmid encoding BI-1-GFP fusion protein (b) or a plasmid encoding Flag-tagged BI-1 protein (c) was transfected into Cos-7 cells. 18 hr after transfection, cells were seeded in chamber slides for fluorescence microscopy. In (a) and (b), cells were analyzed directly using appropriate filters for visualization of the green fluorescence resulting from GFP. In (c), cells were stained with anti-Flag M2 and FITC-conjugated anti-mouse IgG. Cells stained with secondary antibody alone exhibited negligible fluorescence (not shown). Photographs represent ~400 $\times$  original magnification.

(B) BI-1-GFP transfected Cos-7 cells were incubated with the Mitotracker dye before being fixed and visualized by fluorescence confocal microscopy using filters appropriate for the visualization of green (a), red (b), or both (c), resulting from the BI-1-GFP protein and the Mitotracker. Data shown are representative of the majority of doubly stained cells. (C) 293T cells were transiently transfected with either parental vector (Control) or plasmid encoding HA-tagged BI-1. Cells were lysed 2 days later in a hypotonic solution, and crude subcellular fractionations were prepared. Equivalent proportions of each fraction were subjected to SDS-PAGE/immunoblot analysis using antibodies specific for HA-tag, Bcl-2, Bax, F1 $\beta$ -ATPase (mitochondria marker), PARP (nuclear marker), and CPP32 (Caspase-3, cytosolic marker).

Bax. For instance, BI-1 could act upstream of Bax, affecting the production of Bax or the targeting of Bax to its proper intracellular locations. Our data, however, suggest that BI-1 has no significant impact on the levels of Bax or its intracellular location. BI-1 does not appear to be associated with Bax, implying that it does not inhibit Bax directly. However, physical interaction is not necessarily required for mutual antagonism among anti- and proapoptotic Bcl-2 family proteins (Simonian et al., 1996; Tao et al., 1997; Zha and Reed, 1997). Given that the BI-1 associates with Bcl-2 and Bcl-X<sub>L</sub> in mammalian cells, it is also formally possible that BI-1 functions through a Bcl-2 homolog in yeast. We do not favor this hypothesis, mainly because no Bcl-2 homologs are identifiable in *S. cerevisiae* on the basis of amino acid sequences deduced from the complete yeast genome.

BI-1 might function downstream of Bax, based on the apparent scarcity of BI-1 in mitochondrial membranes. In this regard, our preliminary attempts to localize BI-1 indicate that it is predominantly nonmitochondrial and instead may be mostly localized to the ER. In contrast,

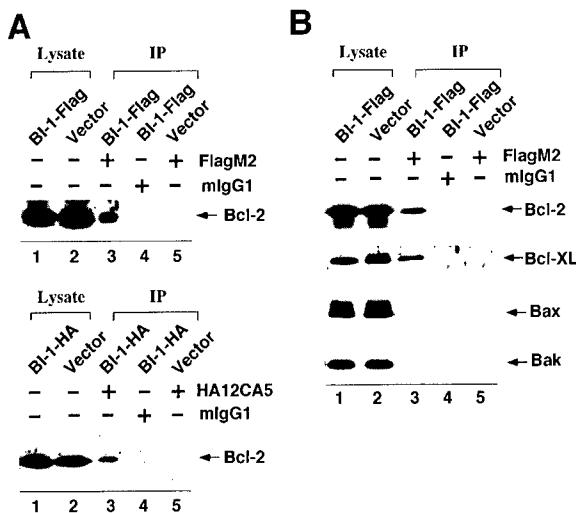


Figure 7. BI-1 Interacts with Bcl-2 in Mammalian Cells

(A) In vivo cross-linking. 293 cells were cotransfected with Bcl-2-encoding plasmid and either a control (vector) or plasmids encoding BI-1-Flag (top panel) or BI-1-HA (bottom panel) proteins. 2 days after transfection, cells were washed in PBS and lysed in RIPA buffer. Immunoprecipitations were performed using normal mouse IgG1 as a negative control or the anti-Flag M2 (top) or anti-HA 12CA5 (bottom) monoclonal antibodies. Immunocomplexes were reduced (to reverse the cross-linking reaction) and analyzed by SDS-PAGE/immunoblotting using anti-Bcl-2 antiserum. Lanes 1 and 2 represent whole cell lysates from cells transfected with Bcl-2 + BI-1 or Bcl-2 + vector, respectively (1/20 of the input for lanes 3-5). Lanes 3-5 were loaded with immune complexes precipitated with the indicated antibodies.

(B) Coimmunoprecipitation. 293 cells were transiently transfected with either Bcl-2-, Bcl-X<sub>L</sub>-, or Bax-encoding plasmids together with either vector control plasmid or BI-1-Flag-encoding plasmid DNA. 2 days later, cells were lysed in 1% NP-40 buffer and immunoprecipitations were performed using either anti-Flag antibody M2 or IgG control. Immune complexes (lanes 3-5) were subjected to SDS-PAGE/immunoblot analysis using antisera specific for Bcl-2, Bcl-X<sub>L</sub>, Bax, or Bak. Whole cell lysates (lanes 1 and 2) are from cells transfected with Bcl-2 (top), Bcl-X<sub>L</sub> (second), Bax (third), or no plasmid (bottom) together with either BI-1-Flag plasmid (lane 1) or vector control (lane 2), representing 1/40 of the input for immunoprecipitation.

Bax has been reported to associate primarily with mitochondria in yeast and mammalian cells (Zha et al., 1996). Thus, to the extent that these two proteins reside in different organelar compartments, these observations support the notion of BI-1 operating downstream of Bax. However, others have shown that when Bcl-2 is targeted exclusively to the ER through substitution of the C terminus of cytochrome *b5*, it retains the ability to block apoptosis induced by some stimuli (Zhu et al., 1996). Moreover, the adenovirus Bcl-2 homolog E1B 19K can antagonize Bax-induced apoptosis but apparently is not associated with mitochondria (White and Cipriani, 1989; Han et al., 1996). This implies that Bcl-2 and E1B 19K need not necessarily be associated with mitochondria where Bax mostly resides, raising the possibility that Bcl-2 and Bax control parallel pathways that independently provide signals for cell survival and death, respectively. If true, then an alternative interpretation is

that BI-1 is a downstream effector of Bcl-2, which prevents Bax-induced death in yeast by substituting for Bcl-2.

Though mitochondria have received much attention recently for their role in apoptosis, the ER has also been implicated in cell death regulation. The ER performs several essential functions, including protein processing and translocation, vesicle transport, and maintaining calcium homeostasis (reviewed by Teasdale and Jackson, 1996). It has been shown that Bcl-2 can alter regulation of Ca<sup>2+</sup> in the ER, preventing loss of Ca<sup>2+</sup> from this organelle following growth factor deprivation and decreasing the basal efflux of Ca<sup>2+</sup> under normal circumstances (Baffy et al., 1993; Lam et al., 1994). Depletion of intra-ER Ca<sup>2+</sup> stores has been reported to induce apoptosis (Baffy et al., 1993). Moreover, release of Ca<sup>2+</sup> into the cytosol can induce mitochondrial permeability transition (PT) pore opening (Bernardi et al., 1994; Kroemer et al., 1996), thus creating a functional connection between the ER and mitochondria. Interestingly, antisense-mediated ablation of one of the ER Ca<sup>2+</sup> channels, namely the inositol triphosphate-gated receptor-1, has revealed a requirement for this Ca<sup>2+</sup> channel in T-cell apoptosis induced by diverse stimuli, including anti-Fas antibodies, glucocorticoids, and ionizing radiation (Jayaraman and Marks, 1997). Thus, regulation of Ca<sup>2+</sup> trafficking through effects on Ca<sup>2+</sup> channels in the ER represents one of several tenable hypotheses for explaining how a multiple-membrane-spanning protein such as BI-1 might directly or indirectly modulate cell death pathways.

What might BI-1 be doing in intracellular membranes? Based on the predicted multiple transmembrane segments, BI-1 could possibly function as a receptor or an ion-channel protein. Similar to ion-channels, some of the putative transmembrane segments of BI-1 when examined on  $\alpha$ -helical wheel plots predict the presence of hydrophilic residues on one face of the  $\alpha$  helices rather than uniformly hydrophobic residues like those found in the transmembrane domains of most cell surface growth factor receptors. If several amphipathic helices assembled in the membrane, creating an aqueous lumen ringed by the hydrophilic surfaces of these putative  $\alpha$  helices, then BI-1 could potentially form an ion channel in membranes. Alternatively, the apparent physical association of BI-1 and Bcl-2 raises the intriguing possibility that BI-1 and Bcl-2 could create heteromeric channels, with Bcl-2 presumably contributing its 5th and 6th amphipathic  $\alpha$ -helical domains, which have been shown to be required for in vitro pore formation (Schenkel et al., 1997), and BI-1 providing some of its amphipathic transmembrane  $\alpha$ -helical segments. Precedent for this idea exists among some types of K<sup>+</sup> channels in which the functional channel is comprised of a heterotetramer, with each subunit contributing transmembrane  $\alpha$  helices that surround a central ion pore (Catterall, 1995). Alternatively, the interaction of Bcl-2 and BI-1 could provide a mechanism by which BI-1 regulates the previously described Bcl-2 channel, or conversely, by which Bcl-2 regulates a hypothetical BI-1 channel, analogous to some voltage-gated Na<sup>+</sup> and Ca<sup>2+</sup> ion channels in which a single protein forms the actual channel but this channel is highly regulated by associated integral

membrane proteins (Catterall, 1995). Also, by functioning as an ion channel, conceivably BI-1 might alter ion gradients, pH, or voltage (DV) across the membranes where Bcl-2 family proteins reside, thus either inhibiting or enhancing their ability to integrate into membranes and form ion channels (review by Reed, 1997a). It should be noted however that Bcl-2 has been reported to bind multiple proteins (Reed, 1997a) and thus may be promiscuous in its interactions with other proteins, including BI-1. Thus, while the significance of the interaction of BI-1 with Bcl-2 remains to be determined, the data presented here nevertheless establish that BI-1 is a novel suppressor of cell death.

The yeast-based functional cloning strategy employed here provides an alternative approach for identifying proteins that regulate mammalian cell death. Though BI-1 can interact with Bcl-2 (or with a complex of proteins that includes Bcl-2), it is unlikely that interaction cloning methods based on either yeast two-hybrid or  $\lambda$ -phage expression library screening using ligand-blotting would have detected BI-1, because of its hydrophobic characteristics. Protein purification attempts based on ability to interact with Bcl-2 also would have been unlikely to succeed for the same reason. Thus, functional screening for Bax suppressors in yeast provides a powerful approach that complements these other methods and that seems likely to yield new insights into the biochemistry and genetics of mammalian cell death regulation. Future studies, including targeted gene knockout experiments in mice, will better define the overall role played by BI-1 in the Bcl-2/Bax pathway for cell death regulation.

#### Experimental Procedures

##### Yeast Methods

Yeast strains used included: BF264-15Dau (*MATa ade1 his2 leu2-3, 112 trp1-1a ura3*) (Lew et al., 1991) and EGY48 (*MAT $\alpha$  trp1 ura3 his3 leu2::plexAop $\epsilon$ -LEU2*). Strain QX95001 is BF264-15Dau containing the *LEU2*-marked mBax-encoding plasmid YEpl51-Bax. Strains BF264-15Dau and EGY48 were maintained in the rich YPD medium, and strain QX95001 was maintained in SD-Leu (synthetic dropout medium lacking leucine). Transformations, plasmid extractions, and protein extracts were prepared as described (Sato et al., 1994; Zha et al., 1996).

For cDNA library screening, QX95001 cells were grown to mid-log phase in the SD-Leu liquid medium and transformed with 100  $\mu$ g of HepG2 cDNA library DNA by a LiOAc method. Bax-resistant transformants were directly selected on galactose-containing synthetic dropout medium lacking leucine and uracil (SD-leu+ura). An aliquot of transformation mixture was also spread on glucose-containing medium to determine transformation efficiency. Bax-resistant colonies were patched onto galactose-containing SD-leu+ura plates. Con-loss assays were performed as described (Ausubel et al., 1991).

##### Plasmid Constructions

pcDNA3-hBax and pRc/CMV-Bcl-2 contain human Bax and human Bcl-2 cDNAs in the expression plasmids pcDNA3 and pRc/CMV, respectively. The C-terminal HA-tagged BI-1 plasmid was constructed in two steps. The C terminus of BI-1 (from the internal BamHI site) was first PCR-amplified to add a Xhol site to the very C terminus of the BI-1 ORF (just before the stop codon). PCR primers used were 5'-GGGGATCCATTGGCCTTCCAG and 5'-GGCTCGA GTTTCTCTCTTCTTCTTATCC. The resulting PCR product was digested with BamHI and Xhol and subcloned into pcDNA3 containing an oligonucleotide encoding three in-frame copies of the HA

tag downstream of the Xhol site, producing plasmid pQX9645. Next, the N-terminal portion of BI-1 was re-ligated into pQX9645, giving rise to pcDNA3-BI-1-HA. An EcoRI-Xhol fragment containing BI-1 cDNA from pcDNA3-BI-1-HA was subcloned into pcDNA3 containing a single copy of the FLAG epitope downstream of the Xhol site and into the GFP vector pEGFP-N2 (Clontech Inc.) between the EcoRI and SalI sites. The 2.6 kbp BI-1 cDNA was subcloned in reverse (antisense) orientation between the Xhol and EcoRI sites of pCL-Neo (Invitrogen, Inc.), producing pCL-Neo-BI-1-AS.

##### Cell Culture and Transfections

293, 293T, GM701, and Cos7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (fetal bovine serum), 1 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin sulfate and transfected as described (Zha et al., 1996). For cell death assays, 293 cells were seeded at  $8 \times 10^5$  cells per 60 mm dish and 1 day later transfected with various combinations of plasmids (9  $\mu$ g total DNA). Precipitates were removed after 8 hr and replaced with fresh medium. Both floating and adherent cells (after trypsinization) were collected at 24 hr post-transfection and analyzed by trypan blue dye exclusion assay, counting a minimum of 300 cells and performing experiments in triplicate (mean value  $\pm$  SD). The transfection efficiency was estimated to be  $\geq 70\%$  based on cotransfections with a  $\beta$ -gal reporter plasmid. DAPI staining for assessing nuclear morphology was also performed.

For GM701 fibroblasts, cells were seeded into 6-well tissue culture dishes at  $6 \times 10^4$  cells/well and cotransfected with 0.5  $\mu$ g of  $\beta$ -gal reporter plasmid and 4  $\mu$ g of various other expression plasmids. Precipitates were removed 6 hr later, and 18 hr posttransfection cells were washed three times with DMEM and incubated in medium containing 0.1% FBS for 30 hr before fixing and staining with X-gal (Zha et al., 1996).

FL5.12 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 10% WEHI-3B conditioned medium, L-glutamine, and antibiotics. Plasmid DNAs (20  $\mu$ g) were introduced into FL5.12 cells by electroporation (GenePulser; Biorad) using 650 V/cm and 1025  $\mu$ F, followed by selection in medium containing puromycin (0.5  $\mu$ g/ml). Clones expressing BI-1-GFP fusion protein were screened initially by fluorescence microscopy, followed by FACS and immunoblot analysis. IL-3 withdrawal and cell viability determinations were performed as described (Wang et al., 1995). For drug-resistance assays, either etoposide (5  $\mu$ g/ml) or staurosporine (0.5  $\mu$ M) was added to cells at a density of  $5 \times 10^5$  cells/ml and incubated for up to 3 days before determining the percentage cell viability based on trypan blue dye exclusion.

##### Immunoprecipitation and Immunoblot Assays

Cell lysates were prepared using HKME solution (10 mM HEPES [pH 7.2], 142 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA) containing 1% NP-40 and protease inhibitors (1 mM PMSF, 5  $\mu$ g/ml leupeptin, 5  $\mu$ g/ml aprotinin, 0.7  $\mu$ g/ml pepstatin) and cleared by incubation with the rec-protein G-Sepharose 4B (ZYMED). Lysates were then diluted in HKME to a final concentration of 0.6% NP-40 and incubated with anti-Flag M2 antibody (Kodak) at 4°C for 2 hr with constant rotating, followed by rec-protein G beads for 1 hr. Immobilized immunocomplexes were washed four times in HKME containing 0.2% NP-40 before boiling in SDS sample buffer. Cell lysates or immunoprecipitates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Biorad). After blocking with 5% non-fat milk, 2% bovine serum albumin (BSA) in TBST (10 mM Tris [pH 7.5]; 142 mM NaCl; 0.1% Tween 20) at the room temperature for 2 hr, blots were incubated in the same solution with various primary antibodies, including monoclonal antibodies specific for HA (12 CA5 [0.8  $\mu$ g/ml]) or Flag (M2 [3  $\mu$ g/ml]) and polyclonal antisera specific for Bcl-2 (0.1% [v/v]), Bcl-X (0.1%), Bax (0.1%), CPP32 (0.1%), PARP (0.1%), or F<sub>1</sub>- $\beta$ -ATPase (0.1%), followed by 0.6  $\mu$ g/ml horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Biorad) secondary antibodies. Bound antibodies were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham).

##### In Vivo Cross-Linking

Transfected 293 cells were washed twice with PBS and then incubated for 20 min at room temperature in PBS with 1 mM DTBP

(dimethyl-3,3'-dithiobispropionimidate [2HCl]) (Pierce, Inc.) with gentle shaking. After extensive washing in PBS, cells were lysed in RIPA (10 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing protease inhibitors, and immunoprecipitations were performed using anti-HA (12CA5) or anti-Flag (M2) monoclonals. Immune complexes were immobilized on protein G beads and washed four times with RIPA, followed by boiling in SDS sample buffer (containing 5%  $\beta$ -mecaptoethanol). Samples were analyzed by SDS-PAGE/immunoblotting using rabbit antisera specific for Bcl-2 or Bax.

#### Immunofluorescence and Confocal Microscopy

Cells transiently transfected with pcDNA3-BI-1-Flag were trypsinized and seeded into chamber slides. The next day, cells were washed in PBS and fixed in PBS containing 3.7% paraformaldehyde, followed by washing twice in PBS. Cells were then permeabilized in 0.1% Triton X-100/PBS for 20 min and preblocked in PBS containing 3% BSA, 2% FBS, 0.1% goat serum. Anti-Flag antibody M2 (3  $\mu$ g/ml) was added to cells in the same solution and incubated for 1 hr, followed by washing three times in PBS/0.1% Triton X-100 and incubation with 2  $\mu$ g/ml FITC-conjugated secondary anti-mouse antibody (Dako) for 1 hr. After washing three times in PBS, slides were covered in Vectashield mounting medium (Vector Laboratories, Inc.) and sealed with nail polish. For two-color analysis, GFP-BI-1-transfected cells were incubated with 20 nM Mitotracker (Molecular Probes, Inc.) in normal growth medium for 20 min at 37°C. Confocal fluorescence microscopy was performed using an Axiohot photomicroscope (Zeiss, Inc., Oberkochen, Germany).

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## Cytoprotection by Bcl-2 Requires the Pore-forming $\alpha 5$ and $\alpha 6$ Helices\*

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We explored whether the putative channel-forming fifth and sixth  $\alpha$ -helices of Bcl-2 and Bax account for Bcl-2-mediated cell survival and Bax-induced cell death in mammalian cells and in the yeast *Saccharomyces cerevisiae*. When  $\alpha 5$ - $\alpha 6$  were either deleted or swapped with each other, the Bcl-2 $\Delta\alpha 5\alpha 6$  deletion mutant and Bcl-2-Bax( $\alpha 5\alpha 6$ ) chimeric protein failed to block apoptosis induced by either Bax or staurosporine in human cells and were unable to prevent Bax-induced cell death in yeast, implying that the  $\alpha 5$ - $\alpha 6$  region of Bcl-2 is essential for its cytoprotective function. Additional experiments indicated that, although  $\alpha 5$ - $\alpha 6$  is necessary, it is also insufficient for the anti-apoptotic activity of Bcl-2. In contrast, deletion or substitution of  $\alpha 5$ - $\alpha 6$  in Bax reduced but did not abrogate apoptosis induction in human cells, whereas it did completely nullify cytotoxic activity in yeast, implying that the pore-forming segments of Bax are critical for conferring a lethal phenotype in yeast but not necessarily in human cells. Bax $\Delta\alpha 5\alpha 6$  and Bax-Bcl-2( $\alpha 5\alpha 6$ ) also retained the ability to dimerize with Bcl-2. Bax therefore may have redundant mechanisms for inducing apoptosis in mammalian cells, based on its ability to form  $\alpha 5$ - $\alpha 6$ -dependent channels in membranes and to dimerize with and antagonize anti-apoptotic proteins such as Bcl-2.

Bcl-2 family proteins play a pivotal role in the regulation of programmed cell death and apoptosis. Some members of this family such as Bcl-2 and Bcl-X<sub>L</sub> function as cell death suppressors, whereas others such as Bax and Bak induce apoptosis (1–3). At least three biochemical characteristics have been ascribed to various Bcl-2 family proteins, including: (a) dimerization with themselves and each other; (b) interactions with other types of proteins, ranging from protein kinases and phosphatases to proteins that bind cell death proteases of the caspase family; and (c) formation of pores or ion channels in membranes (1). The relative significance of these different functions remains to be clarified, but may depend on the precise repertoire of Bcl-2 family proteins expressed in cells and the type of cell death stimuli applied.

The three-dimensional structure of one of the Bcl-2 family proteins, Bcl-X<sub>L</sub>, has been determined, revealing seven  $\alpha$ -helices separated by flexible loops (4). Some other members of the Bcl-2 family, including the anti-apoptotic protein Bcl-2 and the

pro-apoptotic protein Bax, can be readily modeled on the Bcl-X<sub>L</sub> crystallographic coordinates, implying that they share a similar fold despite having opposing effects on cell life and death (5). The C terminus of many Bcl-2 family proteins consists of a stretch of hydrophobic amino acids that serves the purpose of anchoring them within intracellular membranes, particularly the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope, with the bulk of the protein oriented toward the cytosol (6, 7).

Comparisons with other proteins for which structures are available revealed striking structural similarity of Bcl-X<sub>L</sub> to the pore-forming domains of certain bacterial toxins, including: (a) diphtheria toxin, which produces pores for transporting a polypeptide fragment of the toxin across lysosomal/endosomal membranes into the cytosol (8, 9); and (b) the colicins, which form ion channels that kill sensitive *Escherichia coli* by depolarizing their inner membranes (10). Moreover, Bcl-2, Bcl-X<sub>L</sub>, and Bax have been reported to form ion channels in synthetic membranes *in vitro*, when tested under conditions similar to those required for channel formation by diphtheria toxin or the colicins (11–14). However, the characteristics of the channels formed *in vitro* by cytoprotective (Bcl-2, Bcl-X<sub>L</sub>) and cytotoxic (Bax) members of the Bcl-2 family differ. In general, Bcl-2 and Bcl-X<sub>L</sub> tend to form channels having low conductance, display modest cation selectivity, and exist in a mostly closed state, whereas Bax channels typically have 100–1000-fold larger conductances than Bcl-2 or Bcl-X<sub>L</sub> channels, prefer anions, and dwell longer in an open state (reviewed in Ref. 5).

By analogy to structurally similar pore-forming domains from bacterial toxins, the predicted fifth and sixth  $\alpha$ -helices of Bcl-2 and Bax are hypothesized to directly participate in channel formation. These  $\alpha$ -helices are positioned in the core of these proteins (based on models derived from the Bcl-X<sub>L</sub> structure) and are believed to be inserted into the membrane bilayer perpendicular to the membrane surface, with the loop connecting  $\alpha 5$  and  $\alpha 6$  presumably protruding from the other side of the membrane (5). Indeed, deletion of the  $\alpha 5$ - $\alpha 6$  regions from Bcl-2 abolishes its ability to form ion channels in synthetic membranes *in vitro* (12). The structural basis for differences in the channels formed *in vitro* by Bcl-2 and Bax is unknown, but could be due at least in part to differences between the polar residues of the fifth and sixth  $\alpha$ -helices of these proteins. Two acidic amino acids are predicted to be on the hydrophilic face of  $\alpha 5$  in Bcl-2 and Bcl-X<sub>L</sub>, which would presumably line the lumen channel, compared with two basic amino acids in the corresponding position for the pro-apoptotic Bax and Bak proteins (reviewed in Ref. 5). These differences in  $\alpha 5$  and  $\alpha 6$  might account for the relative cation specificity of the Bcl-2 and Bcl-X<sub>L</sub> channels (11, 12), and the anion selectivity of the Bax channel (13).

It remains to be determined whether channels are formed by Bcl-2 family proteins *in vivo* and whether this activity is critical for the biological functions of these proteins. However,

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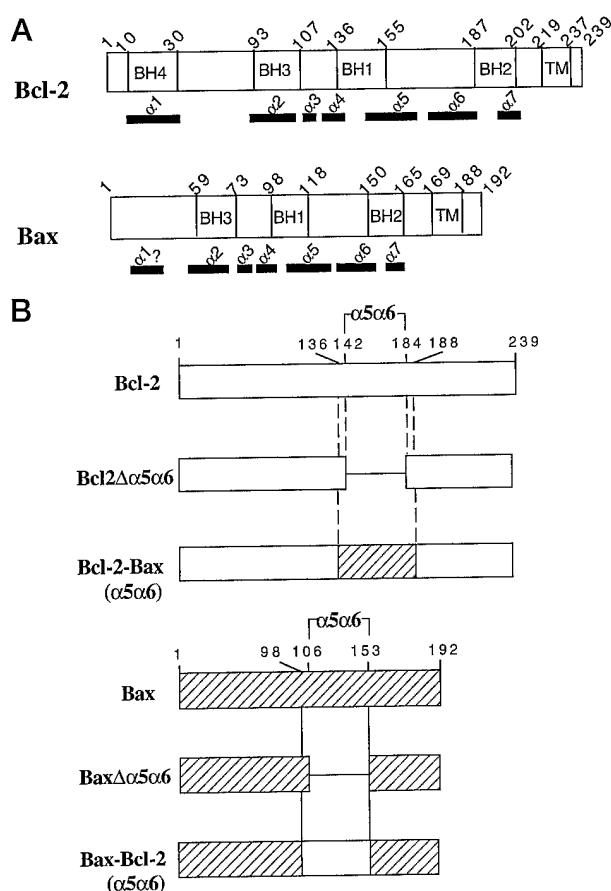
intrinsic bioactivities for the Bcl-2 and Bax proteins have been demonstrated in yeast, where no Bcl-2 homologs apparently exist based on sequence homology searches of the now completed genome of *Saccharomyces cerevisiae*. The Bax and Bak proteins, for example, confer a lethal phenotype when ectopically expressed in either the budding yeast *S. cerevisiae* or the fission yeast *Schizosaccharomyces pombe* (15–21). In contrast, mutants of Bax and Bak that lack the putative pore-forming  $\alpha$ 5 and  $\alpha$ 6 helices are devoid of cytotoxic activity in yeast. Bcl-2 and Bcl-X<sub>L</sub> can rescue yeast from the lethal effects of Bax and Bak, without necessity for dimerization between these proteins (22). Moreover, ectopic expression of Bcl-2 in the absence of Bax or Bak in certain mutant strains of yeast has also been shown to preserve cell viability under some circumstances (23), providing further evidence of an intrinsic function for this anti-apoptotic protein.

In this report, we explored some of the structure-function relations of the Bcl-2 and Bax proteins that may be relevant to their similarity to pore-forming proteins, focusing specifically on the putative pore-forming  $\alpha$ 5 and  $\alpha$ 6 helices. The results provide further insights into the question of why Bcl-2 is cytoprotective and Bax is cytotoxic, and suggest that differences in the  $\alpha$ 5 and  $\alpha$ 6 helices of Bcl-2 and Bax are necessary but insufficient for determining the opposing phenotypes of these proteins.

#### MATERIALS AND METHODS

**Plasmid Constructions**—Human Bcl-2 and human Bax cDNAs were employed as the templates for the mutagenesis experiments. Mutations were created using a two-step polymerase chain reaction method (17, 24). All mutants were initially subcloned between *Eco*RI (5' end) and *Xba*I (3' end) sites in pEG202, pJG4–5, pcDNA3, or pcDNA3-HA plasmids. The following mutagenic primers were used in combination with the wild-type Bcl-2 forward (for pEG202, pJG4–5: 5'-GCGGAATTCA-TGGCGCACGCTGGAGAAC-3'; for pcDNA3: 5'-GCGGAATTGCC-ACCATGGCGCACGCTGGAGAAC-3') and reverse (with C-terminal transmembrane domain (TM): 5'-ATTCTCGAGTCAGTCTCAGAGAC-GATAGG-3'; without TM: 5'-CGCCTCGAGTCAGTCTCAGAGAC-AGCCAGGA-3'), for wild-type Bax forward (for pEG202, pJG4–5, or pcDNA3-HA: 5'-GCGGAATTCATGGACGGGTCGGGGAGGAG-3'; for pcDNA3: 5'-GCGGAATTGCCACATGGACGGGTCGGGGAGGAG-3') and reverse (with TM: 5'-ATTCTCGAGTCAGCCCACATCTTCCA-GAT-3'; without TM: 5'-ATTCTCGAGTCAGGGCGTCCCAAAGTAGG-AGAG-3'), for Bcl-2 $\Delta$  $\alpha$ 5 $\alpha$ 6, 5'-CTGCAACCTGGATCCAGGATAACG-GA-3' (forward) and 5'-CCAGGTGTGCGAGCACCCGTGCTGAAGA-GCTC-3' (reverse), and for Bax2 $\Delta$  $\alpha$ 5 $\alpha$ 6, 5'-GACGGCAACTTCGACCA-GGGTGGTTGGGACGGC-3' (forward) and 5'-GAAGTTGCCGTAGA-AAACATGTCAGC-3' (reverse). For the construction of Bcl-2-Bax or Bax-Bcl-2 chimeras, first a *Sac*I site was introduced into the Bax cDNA by two-step polymerase chain reaction using 5'-GCAGCTGAGCTCTT-TTCTGACGGCAACTTCAAC-3' (forward) and 5'-AGAAAAGAGCTCA-GCTGCCACTCGGAAAAAGAC-3' (reverse) with the above primers for wild-type Bax. Then, the region of the Bcl-2 cDNA and Bax cDNAs between the *Sac*I and *Bam*HI sites was swapped. For the production of recombinant GST-Bax(ΔTM) and GST-Bax $\Delta$  $\alpha$ 5 $\alpha$ 6(ΔTM), cDNAs encoding Bax(ΔTM) and Bax $\Delta$  $\alpha$ 5 $\alpha$ 6(ΔTM) were subcloned between *Eco*RI (5' end) and *Xba*I (3' end) sites in pGEX-4T-1 vector.

**Mammalian Cell Apoptosis Assays**—293T cells were cultured for 12 h in 60-mm diameter dishes in 5 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Fresh medium was exchanged, and 4 h later the cells were co-transfected with 0.5  $\mu$ g of pEGFP (CLONTECH Laboratories, Inc.) and various plasmids encoding wild-type or mutants of Bcl-2 or Bax by a calcium phosphate precipitation method (total amount of DNA normalized to either 1.5 or 2.5  $\mu$ g). Four hours after transfections, fresh medium was exchanged and the cells were cultured for another 20 h before collecting both floating and adherent cells. Half of the recovered cells were used for immunoblot



**FIG. 1. Bcl-2 and Bax mutants and chimeras.** The predicted positions of the  $\alpha$ -helical regions within the human Bcl-2 and Bax proteins are depicted (A), and the  $\alpha$ 5- $\alpha$ 6 region deletion mutants and chimeras of Bcl-2 and Bax are illustrated (B). The boundaries of the regions corresponding to the  $\alpha$ 5 and  $\alpha$ 6 helices were deduced from Ref. 4 and confirmed independently by modeling the human Bcl-2 and Bax proteins on the Bcl-X<sub>L</sub> coordinates (5). Numbers indicate amino acid positions. The open bars refer to Bcl-2 and the hatched bars to Bax.

assays, and the remainder were stained with 4',6-diamidino-2-phenylindole to determine the percentages of GFP-positive cells with apoptotic nuclei (25).

GM701 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum. Cells were transfected with pRC/CMV-hBcl2, pcDNA3-Bcl-2 $\Delta$  $\alpha$ 5 $\alpha$ 6, or pcDNA3-Bcl-2-Bax(α5α6) by a calcium-phosphate precipitation method and selected in 1.4 mg/ml (active) G418. Pools of stable transfectants were passaged and then were cultured in 96-well plates for 12 h at a density of  $1 \times 10^4$  cells/0.1 ml/well. Fresh medium was exchanged, and 1  $\mu$ M staurosporine (STS) was added to induce apoptosis. After 24 h, cell viability was determined by trypan blue dye exclusion assay.

**Yeast Cytotoxicity Assays**—EGY48 strain cells were transformed by the lithium acetate method, using 1  $\mu$ g of plasmid DNA (25, 26). Cells were then plated on histidine-deficient glucose-based minimal medium supplemented with other essential amino acids. Colonies were counted after culturing at 30 °C for 3 days. For the examination of Bcl-2-mediated rescue of yeast from Bax-induced cell death, EGY48 cells were co-transformed with 1  $\mu$ g of pGilda-Bax and 1  $\mu$ g of pJG4–5-Bcl-2, pJG4–5-Bcl-2 $\Delta$  $\alpha$ 5 $\alpha$ 6, pJG4–5-Bcl-2-Bax(α5α6), or pJG4–5-Bax-Bcl-2(α5α6), and plated on both histidine- and tryptophan-deficient glucose-based medium to select for the plasmids. Single colonies of transformed yeast cells were re-streaked on galactose-containing medium to induce the GAL-1 promoters in these plasmids and cultured for 4 days (25).

**Yeast Two-hybrid Assays**—Protein-protein interactions were evaluated by yeast two-hybrid assay as described previously, using EGY48 cells either for *LEU2* or *lacZ* reporter gene assays, in conjunction with pEG202 (LexA DNA-binding domain) and pJG4–5 (B42 transactivation domain) plasmids (15, 17, 27). Growth on leucine-deficient medium was scored 4 days after spotting on minimal medium plates containing 2% galactose and 1% raffinose to induce expression of the transactivation

<sup>1</sup> The abbreviations used are: TM, transmembrane; DOPC, 1,2-dioleoylphosphatidylcholine; DOPG, 1,2-dioleoylphosphatidylglycerol; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; STS, staurosporine.

domain-containing proteins from the GAL1 promoter in pJG4-5. Filter assays were similarly performed for  $\beta$ -galactosidase measurements, using cells plated on either galactose- or glucose-containing minimal medium supplemented with leucine. Blue color development was scored at 2 h after adding 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal).

**Immunoprecipitation and Immunoblotting Assays**—For co-immunoprecipitation experiments, 293T cells ( $2 \times 10^6$ ) were cultured for 12 h in 10 ml of medium. Fresh medium was exchanged, and 4 h later the cells were co-transfected with 10  $\mu$ g of pRC/CMV-Bcl-2 and 10  $\mu$ g of pcDNA3-HA-Bax, pcDNA3-HA-Bax $\Delta\alpha 5\alpha 6$ , or pcDNA3-HA-Bax-Bcl-2( $\alpha 5\alpha 6$ ), or with 10  $\mu$ g of pcDNA3-Bax and 10  $\mu$ g of pRC/CMV-Bcl-2, pcDNA3-Bcl-2 $\Delta\alpha 5\alpha 6$ , or pcDNA3-Bcl-2-Bax( $\alpha 5\alpha 6$ ), by a calcium phosphate precipitation method. Four hours after transfections, fresh medium was exchanged and the cells were cultured for another 4 h before lysing in 0.6 ml of Nonidet P-40 lysis buffer (10 mM Hepes (pH 7.5) 142.5 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.2% Nonidet P-40), containing 1 mM phenylmethylsulfonyl fluoride, 5  $\mu$ g/ml leupeptin, and 5  $\mu$ g/ml aprotinin. After pre-clearing with 50  $\mu$ l of Protein G-Sepharose at 4 °C for 1 h, immunoprecipitations were performed by incubating 0.2 ml of lysate with 20  $\mu$ l of Protein G-Sepharose preabsorbed with 5  $\mu$ g of anti-Bcl-2 mouse monoclonal antibody ascites (clone 4D7) or 10  $\mu$ l of anti-Bax rabbit serum at 4 °C for 2 h (28, 29). After extensive washing in Nonidet P-40 lysis buffer, beads were boiled in 60  $\mu$ l of Laemmli buffer and 20  $\mu$ l of the eluted proteins were subjected to SDS-PAGE (12%) immunoblot analysis using anti-HA mouse monoclonal antibody conjugated with horseradish peroxidase (Boehringer Mannheim) or 4D7 anti-Bcl-2 mouse monoclonal antibody. For detection of Bcl-2, horseradish peroxidase-conjugated anti-mouse (Bio-Rad) antibody was employed. Immunodetection was achieved by using an enhanced chemiluminescence system (Amersham Pharmacia Biotech) with exposure to x-ray film.

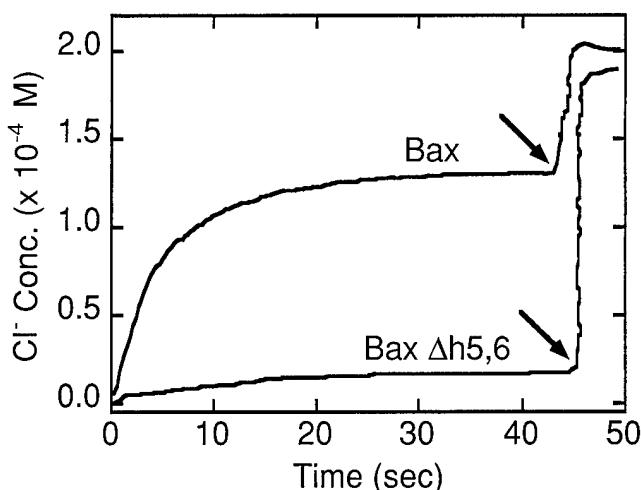
For immunoblot assays, whole cell lysates were normalized for total protein content, and immunoblot assays were performed as described previously using 0.1% (v/v) anti-LexA rabbit serum or either anti-Bax or anti-Bcl-2 rabbit serum (21, 29).

**Ion Channel Assays**—Recombinant GST-Bax ( $\Delta$ TM) and GST-Bax $\Delta\alpha 5\alpha 6$  ( $\Delta$ TM) proteins were produced from pGEX-4T-1 in *E. coli* (BL21 (DE3) strain) bacteria and purified by glutathione-Sepharose affinity chromatography essentially as described (12, 30, 31). GST was removed by cleavage with thrombin, and the Bax( $\Delta$ TM) and Bax $\Delta\alpha 5\alpha 6$  ( $\Delta$ TM) proteins were subsequently purified by ion-exchange chromatography (12, 30, 31) and dialyzed into 20 mM Tris-HCl, pH 8.0. Folding of the purified proteins was confirmed by circular dichroism measurements carried out on an AVIV 60DS spectropolarimeter. Proteins were assayed for channel activity on KCl-loaded unilamellar liposomes composed of 60% DOPC (1,2-dioleoylphosphatidylcholine) and 40% DOPG (1,2-dioleoylphosphatidylglycerol) at pH 4.0, measuring Cl<sup>-</sup> ion efflux as described (12).

## RESULTS

To examine the biological significance of the putative pore-forming  $\alpha 5$  and  $\alpha 6$  helices within Bcl-2 and Bax, mutants having  $\alpha 5$  and  $\alpha 6$  deleted were prepared. Alternatively, the  $\alpha 5$  and  $\alpha 6$  helices were swapped, thus generating chimeric proteins in which the  $\alpha 5$  and  $\alpha 6$  helices of Bax were replaced with those from Bcl-2 and *vice versa* (Fig. 1).

Previously, we demonstrated that deletion of the  $\alpha 5\alpha 6$  region from Bcl-2 abolishes the ability of the recombinant protein to form pH-dependent channels in liposomes *in vitro* (12). To explore the relevance of the  $\alpha 5\alpha 6$  region of Bax to its *in vitro* channel activity, recombinant Bax and Bax $\Delta\alpha 5\alpha 6$  proteins were produced in bacteria (without their C-terminal hydrophobic domains ( $\Delta$ TM) for solubility purposes) and purified (data not shown). When applied at ~150 ng/ml to KCl-loaded unilamellar liposomes under conditions previously shown to be permissive for channel formation by Bcl-2 family proteins (11–14), Bax ( $\Delta$ TM) induced striking ion efflux (Fig. 2). In contrast, the Bax $\Delta\alpha 5\alpha 6$  ( $\Delta$ TM) protein exhibited little or no channel activity under the same conditions. Additional experiments revealed that Bax channel formation was dependent on acidic pH (optimal pH ~4.0) and the presence of acidic lipids within liposomes (DOPG), consistent with prior studies of Bax and other Bcl-2 family proteins (11–14). In contrast, the Bax $\Delta\alpha 5\alpha 6$

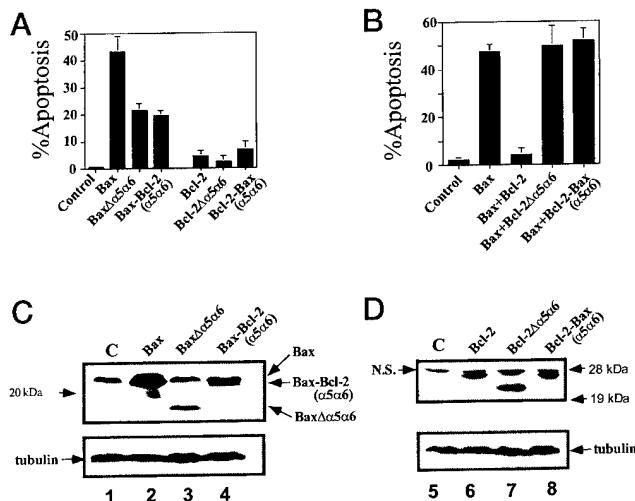


**Fig. 2. Bax channel activity requires  $\alpha 5\alpha 6$  region.** Bax ( $\Delta$ TM) or Bax $\Delta\alpha 5\alpha 6$  ( $\Delta$ TM) were added at 150 ng/ml to KCl-loaded unilamellar liposomes composed of 40:60 (mol/mol) DOPG:DOPC at pH 4.0 and ion efflux was monitored using a Cl<sup>-</sup> electrode as described previously (12). Triton X-100 (0.1%) was added to release residual KCl at the point indicated by arrow.

( $\Delta$ TM) protein induced either negligible ion-efflux or (at higher concentrations) exhibited only nonspecific effects, producing similar amounts of Cl<sup>-</sup> release at both neutral and acidic pH and regardless of whether liposomes contained acidic lipids (DOPG) or were composed entirely of neutral lipids (DOPC) (data not shown). Although the absence of the C-terminal membrane anchoring domain may reduce the efficiency, these experiments nevertheless demonstrate  $\alpha 5\alpha 6$ -dependent channel formation by Bax *in vitro*.

**Studies of Bcl-2 and Bax Mutants in Mammalian Cells**—When expressed in the human kidney epithelial cell line 293T by transient transfection, the wild-type Bax protein induced apoptosis in nearly half of the successfully transfected cells, as determined by 4',6-diamidino-2-phenylindole staining of GFP-expressing cells (Fig. 3). Similarly, apoptosis was also induced by transfection with plasmids encoding either the Bax $\Delta\alpha 5\alpha 6$  or Bax-Bcl-2( $\alpha 5\alpha 6$ ) proteins into 293T cells. The Bax $\Delta\alpha 5\alpha 6$  and Bax-Bcl-2( $\alpha 5\alpha 6$ ) proteins consistently induced a lower percentage of the transiently transfected 293T cells to undergo apoptosis when compared with wild-type Bax in experiments where varying amounts of these plasmid DNAs were employed (1, 2, 4, and 8  $\mu$ g). However, immunoblot analysis of lysates prepared from the transfected 293T cells suggested that these mutant proteins may be produced at somewhat lower levels than the wild-type Bax protein (Fig. 3C; data not shown). These results indicate that the  $\alpha 5$  and  $\alpha 6$  helices of Bax are not absolutely required for apoptosis induction in 293T cells. Furthermore, introduction of the  $\alpha 5$  and  $\alpha 6$  helices from Bcl-2 into the Bax protein is insufficient to convert Bax from a killer to a protector protein.

The bioactivities of Bcl-2 mutant proteins lacking either  $\alpha 5$  and  $\alpha 6$  (Bcl-2 $\Delta\alpha 5\alpha 6$ ) or which contained the corresponding  $\alpha 5\alpha 6$  region from Bax (Bcl-2-Bax( $\alpha 5\alpha 6$ )) were compared against the wild-type Bcl-2 protein in transient co-transfection assays to determine whether these proteins could suppress apoptosis induced by Bax. In contrast to wild-type Bcl-2, transfections performed with plasmids encoding the Bcl-2 $\Delta\alpha 5\alpha 6$  or Bcl-2-Bax( $\alpha 5\alpha 6$ ) proteins failed to suppress Bax-induced apoptosis in 293T cells (Fig. 3B). Immunoblot analysis of lysates prepared from these transiently transfected cells revealed at least comparable levels of production of the Bcl-2 $\Delta\alpha 5\alpha 6$  and Bcl-2-Bax( $\alpha 5\alpha 6$ ) proteins compared with wild-type Bcl-2 (Fig. 3D). Thus, removal of the  $\alpha 5\alpha 6$  region from Bcl-2 or replace-



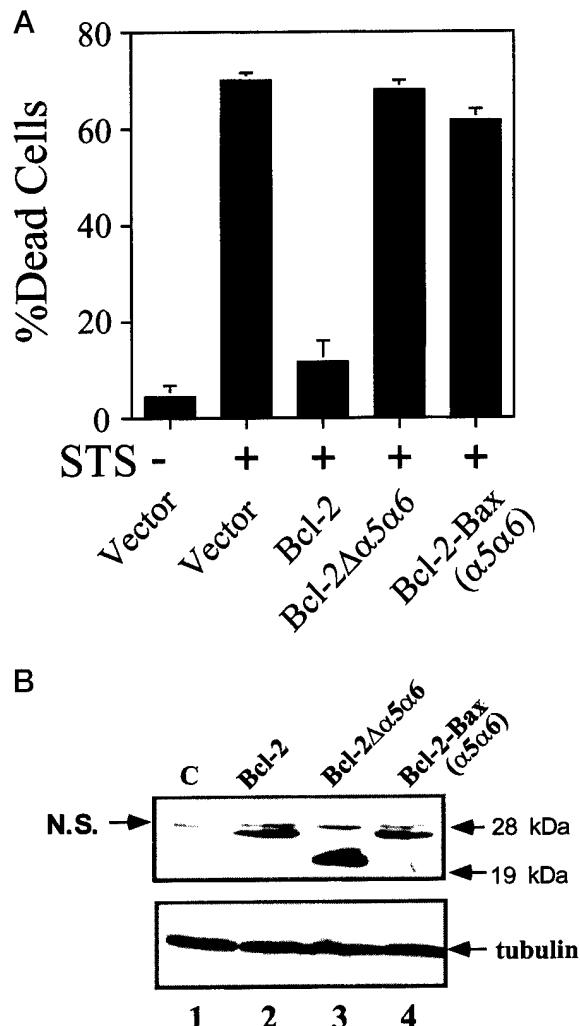
**FIG. 3. Function and expression of Bax and Bcl-2 mutant proteins in 293T cells.** *A*, 293T cells were transiently transfected with 0.5  $\mu$ g of pEGFP and 1  $\mu$ g each of plasmids encoding the indicated proteins. Cells were collected 24 h after transfection, and the percentage of GFP-positive cells with apoptotic nuclei was determined by 4',6-diamidino-2-phenylindole staining (mean  $\pm$  S.D.;  $n = 3$ ). *Control*, pcDNA3. *B*, 293T cells were transiently transfected with 0.5  $\mu$ g of pEGFP and 2  $\mu$ g total of the plasmids described below. *Control*, pcDNA3 (2  $\mu$ g); *Bax*, pcDNA3-Bax (1  $\mu$ g) and pcDNA3 (1  $\mu$ g); *Bax+Bcl-2*, pcDNA3-Bax (1  $\mu$ g) and pRC/CMV-Bcl-2 (1  $\mu$ g); *Bax+Bcl-2Δα5α6*, pcDNA3-Bax (1  $\mu$ g) and pcDNA3-Bcl-2Δα5α6 (1  $\mu$ g); *Bax+Bcl-2-Bax(Δ5α6)*, pcDNA3-Bax (1  $\mu$ g) + pcDNA3-Bcl-2-Bax(Δ5α6) (1  $\mu$ g). The percentage of apoptotic GFP-expressing cells is indicated (mean  $\pm$  S.D.;  $n = 3$ ). *C* and *D*, immunoblot analysis of lysates prepared from 293T cells transfected as in *A* and *B*. All samples were normalized for total protein content (20  $\mu$ g/lane). The blot in *C* (lanes 1–4) was probed with anti-Bax antiserum, whereas anti-Bcl-2 antiserum was employed for the blot in *D* (lanes 5–8). Blots were also incubated with anti-tubulin antibody to confirm loading of equivalent amounts of intact protein (lower panel). *N.S.* indicates non-specific band.

ment of the corresponding region from Bax abolishes the ability of Bcl-2 to block Bax-mediated apoptosis. When expressed in 293T cells without co-transfection of Bax, neither the Bcl-2Δα5α6 nor the Bcl-2-Bax(Δ5α6) protein induced significant apoptosis (Fig. 3A), arguing that substitution of the α5-α6 region of Bax does not convert Bcl-2 into a killer protein.

To further explore the function of the Bcl-2Δα5α6 and Bcl-2-Bax(Δ5α6) proteins, their ability to inhibit STS-induced apoptosis in GM701 cells was compared with the wild-type Bcl-2 protein. Treatment with this broad-specificity kinase inhibitor induced apoptosis in ~70% of GM701 cells (Fig. 4). Wild-type Bcl-2 potently suppressed STS-induced apoptosis. In contrast, neither Bcl-2Δα5α6 nor Bcl-2-Bax(Δ5α6) interfered with STS-induced apoptosis (Fig. 4A), despite expression of these mutant proteins at levels equivalent to or greater than the wild-type Bcl-2 protein (Fig. 4B). Expression of Bcl-2Δα5α6 or Bcl-2-Bax(Δ5α6) in GM701 cells did not induce apoptosis in the absence of STS, indicating that these Bcl-2 mutant proteins are not intrinsically cytotoxic (data not shown). Taken together, these observations indicate that the α5-α6 region of Bcl-2 is essential for its anti-apoptotic activity in mammalian cells.

**Studies of Bcl-2 and Bax Mutant Proteins in Yeast**—Ectopic expression of Bax in *S. cerevisiae* has been shown to induce cell death through a Bcl-2-suppressible mechanism (15, 17, 21). The behavior of the Bcl-2 and Bax mutants was therefore tested in budding yeast. As in our prior reports (15, 17, 21, 25), wild-type and mutant versions of Bcl-2 and Bax were expressed as LexA fusion proteins, using the LexA sequences as an epitope tag for monitoring expression levels.

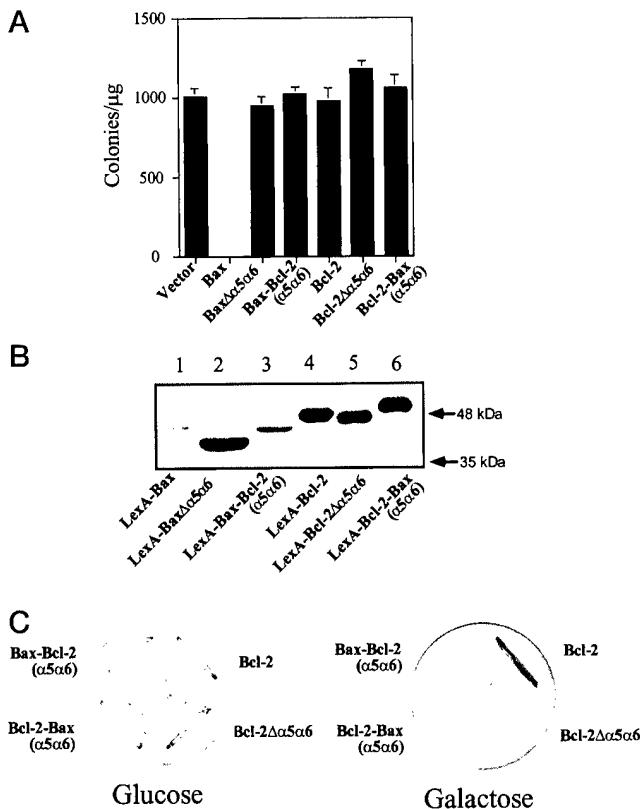
In contrast to the results obtained in mammalian cells, deletion of the α5-α6 region from Bax or substitution of the



**FIG. 4. Functional analysis of Bcl-2 mutants in GM701 cells.** *A*, stable transfectants of GM701 cells were prepared by selection in G418 after transfection with the following plasmids: pcDNA3 (*Vector*), pRC-CMV-Bcl-2, pcDNA3-Bcl-2Δα5α6, and pcDNA3-Bcl-2-Bax(Δ5α6). Cells were cultured at  $1 \times 10^4$  cells/0.1 ml/well in a 96-well dish and treated with or without STS (1  $\mu$ M) for 24 h before assessing viability by trypan blue dye exclusion. *B*, immunoblot analysis of lysates prepared from stably transfected GM701 cells was performed, after normalization for total protein content (20  $\mu$ g/lane). Blots were incubated with either anti-Bcl-2 antiserum (top panel) or anti-tubulin antibody (lower panel). *N.S.* indicates non-specific band.

corresponding region from Bcl-2 abolished the cell death-inducing activity of Bax in yeast, as determined by a colony-forming assay, which measures relative numbers of viable clonogenic cells (15, 17, 21). As shown in Fig. 5A, yeast transformed with the plasmid encoding wild-type Bax formed very few colonies due to the lethal effect of Bax expression, whereas numerous colonies (typically  $>1000/\mu$ g of plasmid DNA) were formed when yeast were transformed with plasmids encoding BaxΔα5α6 or Bax-Bcl-2(Δ5α6). The failure of BaxΔα5α6 and Bax-Bcl-2(Δ5α6) to kill yeast was not attributable to poor expression of these proteins, as revealed by immunoblot assays performed using cells that had been cotransformed with Bcl-2 to nullify the cytotoxic actions of the wild-type Bax protein (Fig. 5B). Thus, the α5-α6 region of Bax is required for its cytotoxic activity in *S. cerevisiae*.

Although necessary for inducing yeast cell death, the α5-α6 helices of Bax are insufficient for mediating the lethal effects of Bax because the chimeric Bcl-2-Bax(Δ5α6) protein, in which the α5-α6 of Bax had been substituted for the corresponding



**FIG. 5. Functional analysis of Bax and Bcl-2 mutants in yeast.** A, EGY48 strain yeast were transformed with 1  $\mu$ g of either pEG202 parental DNA (Vector) or pEG202 plasmids containing cDNAs encoding the indicated proteins. Cells were plated on plasmid-selective medium (histidine-deficient). The number of independent colonies was counted after 3 days (mean  $\pm$  S.D.;  $n = 3$ ). B, lysates were prepared from EGY48 cells transformed as in A, normalized for total protein content (20  $\mu$ g), and then subjected to SDS-PAGE (7.5%) immunoblot assay using anti-LexA antiserum for detection of either the LexA DNA fragment encoded in the pEG202 parental vector or LexA fusion proteins containing Bcl-2, Bax, or various Bax mutants as indicated. C, yeast cells were co-transformed with 1  $\mu$ g of pGilda-Bax and 1  $\mu$ g of pJG4-5 plasmids encoding wild-type Bcl-2, Bcl-2Δα5α6, Bcl-2-Bax(α5α6), or Bax-Bcl-2(α5α6). These plasmids utilize the GAL1 promoter to drive gene expression, resulting in repression of expression when cells are grown on glucose plates and gene induction on galactose. Transformants were first allowed to grow on histidine-deficient glucose plates (left) and then restreaked on galactose plates (right). Photographs represent growth obtained after culture at 30 °C for 4 days.

region within the Bcl-2 protein, failed to display a lethal phenotype in yeast (Fig. 5A). Immunoblot analysis again confirmed production of this protein at levels equivalent to or greater than wild-type Bax (Fig. 5B), discounting poor expression as an explanation of the findings. Taken together, these observations indicate that the putative pore-forming  $\alpha$ 5 and  $\alpha$ 6 helices of Bax are necessary but insufficient for conferring a lethal phenotype in *S. cerevisiae*.

Bcl-2 can rescue yeast from the lethal effects of the wild-type Bax protein (15, 17, 21). To explore the role of the  $\alpha$ 5- $\alpha$ 6 region of Bcl-2 for abrogation of Bax-induced cell death in yeast, cDNAs encoding wild-type or mutant Bcl-2 proteins were subcloned into a plasmid pJG4-5 in which expression is driven from a conditional GAL1 promoter. These galactose-inducible plasmids were then co-transformed into yeast with pGilda-Bax, which also expresses wild-type Bax by GAL1 promoter, and the cells were plated initially on glucose to repress the GAL1 promoter. The resulting transformants were then streaked onto either glucose (control) or galactose (test) plates. As shown in Fig. 5C, wild-type Bcl-2 effectively rescued yeast from the lethal effects of Bax, allowing growth of cells on galactose plates,

**TABLE I**  
Summary of two-hybrid assay results

Binding results were deduced from yeast two-hybrid assays performed in both directions. Each cDNA was subcloned into pEG202 (for LexA-fusion) and pJG4-5 (for B42-fusion) vectors. Interactions were designated as "+" only if both directions showed positive binding signals (both  $\beta$ -galactose activity and Leu(-) assay) in comparison with negative control based on LexA-Fas and B42-Ras.

	Bcl-2	Bcl-2Δα5α6	Bcl-2-Bax(α5α6)	Bax	BaxΔα5α6	Bax-Bcl-2(α5α6)
Bcl-2	+	+	+	+	+	+
Bcl-2Δα5α6	+	-	-	-	-	-
Bcl-2-Bax(α5α6)	+	-	-	$\pm$	+	+
Bax	+	-	$\pm$	+	+	+
BaxΔα5α6	+	-	+	+	- <sup>a</sup>	+
Bax-Bcl-2(α5α6)	+	-	+	+	+	+

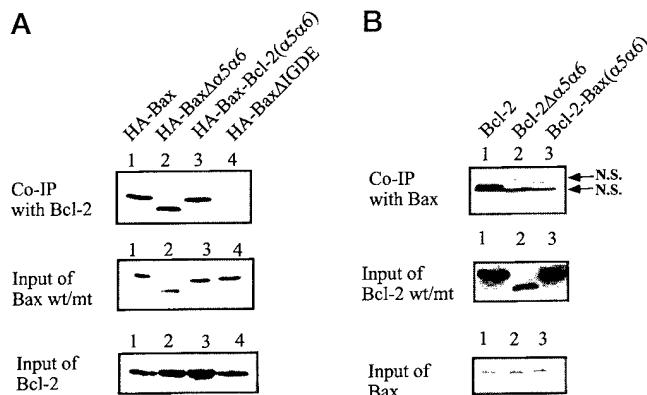
<sup>a</sup> LexA-BaxΔα5α6 showed relatively high background and the signal of LexA-BaxΔα5α6 and B42-BaxΔα5α6 was similar to the negative control of LexA-Bax Δα5α6 and B42-Ras, thus resulting in a "-" score.

whereas the Bcl-2Δα5α6 mutant lacking the putative pore-forming  $\alpha$ 5 and  $\alpha$ 6 helices and the Bcl-2-Bax(α5α6) chimera containing the  $\alpha$ 5- $\alpha$ 6 region from Bax failed to nullify Bax-induced yeast cell death. Immunoblot analysis confirmed expression of these Bcl-2 mutant proteins at levels comparable to the normal Bcl-2 protein (data not shown). Thus, the  $\alpha$ 5- $\alpha$ 6 region of Bcl-2 appears to be necessary for rescuing yeast from the cytotoxic actions of Bax. However, the putative pore-forming  $\alpha$ -helices of Bcl-2 are evidently insufficient for rescue, as expression of the Bax-Bcl-2(α5α6) in which the  $\alpha$ 5- $\alpha$ 6 region of Bcl-2 was inserted in place of the corresponding segment of Bax also failed to protect yeast from Bax-induced cell death (Fig. 5C). Thus, similar to the results obtained in mammalian cells, the  $\alpha$ 5- $\alpha$ 6 region of Bcl-2 appears to be necessary but insufficient for the cytoprotective effect of Bcl-2.

**Analysis of Dimerization Capabilities of Bcl-2 and Bax Mutants**—Bcl-2 and Bax are known to both homodimerize with themselves and heterodimerize with each other (1-3). We explored the effects of deleting the  $\alpha$ 5- $\alpha$ 6 regions of Bcl-2 and Bax or swapping them on homo- and heterodimerization, using a yeast two-hybrid approach (Table I). For these assays, mutant and wild-type Bcl-2 and Bax proteins were expressed with appended N-terminal LexA DNA binding or B42 transactivation domains, but without their C-terminal membrane anchoring regions which could interfere with nuclear import. Removal of the membrane anchoring domain from the C terminus of Bax also abolishes its insertion into mitochondrial membranes, abolishing entirely or greatly reducing its cytotoxicity in yeast (11-13).

The BaxΔα5α6, Bax-Bcl-2(α5α6), and wild-type Bax proteins retained the ability to interact with both Bcl-2 and Bax in yeast two-hybrid assays, consistent with reports indicating the ability of the second  $\alpha$ -helix (BH3 domain) within this protein to bind to pockets found on the surface of other Bcl-2 family proteins (32). Thus, the  $\alpha$ 5- $\alpha$ 6 region of Bax is not required for dimerization with the wild-type Bcl-2 or Bax proteins. The Bax-Bcl-2(α5α6) chimeric protein also retained the ability to interact with itself (Table I), implying that its lack of cell death inducing activity in yeast cannot be attributed to defective homodimerization. In contrast, the BaxΔα5α6 protein failed to interact with itself, consistent with structural studies that have implicated portions of the  $\alpha$ 5 and  $\alpha$ 6 helices in forming the base of the pocket into which the BH3 domain inserts (32).

Analysis of the  $\alpha$ 5- $\alpha$ 6 region mutants of Bcl-2 revealed that all retained the ability to interact with Bcl-2 in yeast two-hybrid assays, implying that they were not grossly misfolded despite their apparent lack of bioactivity in both yeast and



**Fig. 6. Analysis of dimerization of Bax and Bcl-2 mutant proteins by co-immunoprecipitation.** *A*, 293T cells were transfected with pRc-CMV-Bcl2 (lanes 1–4) and pcDNA3-HA-Bax (lane 1), pcDNA3-HA-BaxΔα5α6 (lane 2), pcDNA3-HA-Bax-Bcl-2(α5α6) (lane 3), or pcDNA3-HA-BaxΔIGDE (lane 4). Two days after transfection, cell lysates were prepared and subjected to immunoprecipitation using anti-Bcl-2 monoclonal antibody. Immune complexes were analyzed by SDS-PAGE (12%) immunoblot assay, using anti-HA antibody (upper panel) or anti-Bcl-2 antiserum for immunodetection (bottom panel). Middle panel represents lysates from the same cells that were normalized for total protein content (20  $\mu$ g/lane) and analyzed for relative levels of Bax protein by immunoblotting using anti-Bax antiserum, confirming production of wild-type Bax and all Bax mutants in the transfected cells. *B*, 293T cells were transiently transfected with pcDNA3-Bax (lanes 1–3) and pRc/CMV-h Bcl-2 (lane 1), pcDNA3-Bcl-2Δα5α6 (lane 2), or pcDNA3-Bcl-2-Bax(α5α6) (lane 3). Immunoprecipitations were performed using anti-Bax antiserum, followed by SDS-PAGE (12%) immunoblot analysis using anti-Bcl-2 monoclonal antibody (upper panel) or anti-Bax antiserum (bottom panel) for detection. In the middle panel, lysates were normalized for total protein content (20  $\mu$ g) and subjected to immunoblot assay using anti-Bcl-2 antiserum, confirming production of wild-type Bcl2 and all Bcl-2 mutant proteins. Negative controls to confirm the specificity of these protein interactions included immunoprecipitations performed with HA-BaxΔIGDE and anti-HA antibodies and blotting with anti-Bcl-2 antibody (data not shown).

mammalian cells. However, neither Bcl-2Δα5α6 nor Bcl-2-Bax(α5α6) homodimerized. The Bcl-2Δα5α6 deletion mutant also entirely failed to interact with Bax, and the Bcl-2-Bax(α5α6) chimera displayed reduced interaction with Bax in two-hybrid assays compared with the wild-type Bcl-2 protein (Table I).

To further explore the dimerization capabilities of the α5-α6 region mutants of Bcl-2 and Bax, co-immunoprecipitation experiments were performed using lysates from 293T cells that had been transiently transfected with plasmids encoding these proteins (Fig. 6). Consistent with the results of yeast two-hybrid experiments, HA-epitope tagged versions of the wild-type and α5-α6 region mutants of Bax retained the ability to co-immunoprecipitate with Bcl-2, and were recovered in anti-Bcl-2 immune complexes with approximately equivalent efficiency relative to each other (Fig. 6). As a control, experiments were also performed with a mutant of Bax in which a well conserved I-G-D-E amino acid sequence found within the BH3 domain had been deleted. Consistent with our previous studies of this mutant, no co-immunoprecipitation with Bcl-2 was detected, thus confirming the specificity of the results (24).

Analysis of the Bcl-2 mutants also reinforced the findings of yeast two-hybrid assays. When expressed in 293T cells, the Bcl-2Δα5α6 deletion mutant failed to co-immunoprecipitate with Bax and the Bcl-2-Bax(α5α6) chimeric protein displayed reduced co-immunoprecipitation relative to the wild-type Bcl-2 proteins. Immunoblot analysis of the same lysates indicated that the wild-type Bcl-2 and Bcl-2-Bax(α5α6) proteins were produced at comparable levels in transiently transfected 293T cells, excluding differences in the relative amounts of these

proteins as a likely explanation for the reduced ability of Bcl-2-Bax(α5α6) to co-immunoprecipitate with Bax (Fig. 6). A variety of control co-immunoprecipitations using HA-tagged or untagged irrelevant proteins were performed, confirming the specificity of the results presented in Fig. 6 (data not shown).

## DISCUSSION

Bcl-2 and Bax are known to form ion channels in synthetic membranes *in vitro*, and it has been speculated that the regions predicted to coincide with the α5 and α6 helices of the homologous protein Bcl-X<sub>L</sub> are directly involved in this process (5). Here, we report the results of experiments in which the predicted α5 and α6 region of Bcl-2 and Bax were either deleted or swapped with each other. Our data provide evidence that: (a) α5 and α6 of Bcl-2 are required for its cytoprotective activity in both mammalian cells and yeast, (b) α5 and α6 of Bax are necessary for its cytotoxic activity in yeast but not in mammalian cells, and (c) swapping the α5-α6 regions of Bcl-2 and Bax is insufficient for converting the phenotype of Bcl-2 to a killer and Bax to a protector. These results imply that, although necessary, these α-helices are apparently insufficient to explain why Bcl-2 is anti-apoptotic and Bax is pro-apoptotic in most cellular contexts.

The observation that the α5-α6 region is not required for Bax-induced apoptosis in mammalian cells can presumably be explained by the ability of its BH3 domain (predicted second α-helix) to bind to and antagonize anti-apoptotic Bcl-2 family proteins (27). As shown here, the α5-α6 mutants of Bax retained the ability to co-immunoprecipitate with Bcl-2 and to interact with Bcl-2 in yeast two-hybrid assays. Previous studies have shown that overexpressing fragments of Bax or Bak that retain little more than their BH3 domain are sufficient to bind Bcl-2 or Bcl-X<sub>L</sub> and to induce apoptosis in mammalian cells (33). Similarly, a Bcl-2 family subgroup comprising pro-apoptotic proteins such as Bik, Bid, Bim, and Hrk has sequence similarity with other family members that is limited to the BH3 domain. Predicted structures for these proteins cannot be modeled on the Bcl-X<sub>L</sub> coordinates, implying that do not share structural similarity with the ion channel-forming proteins such as Bcl-2, Bcl-X<sub>L</sub>, and Bax (11–13). This BH3-mediated cell death mechanism may be relevant only in cells that express anti-apoptotic members of the Bcl-2 family, accounting for why α5-α6 region mutants of Bax were inactive in yeast that lack an identifiable Bcl-2 family protein. However, the observation that deletion of the α5-α6 region of Bax abrogates its cytotoxic function in yeast raises the possibility that Bax has two mechanisms for inducing apoptosis in mammalian cells: one that relies on BH3-mediated antagonism of proteins such as Bcl-2 and Bcl-X<sub>L</sub> and another that maps to the α5 and α6 helices required for its channel-forming activity. Support for a second, BH3-independent mechanism of cell killing has been obtained through experiments involving BH3 domain mutants of Bax that failed to dimerize with Bcl-2 or Bcl-X<sub>L</sub>, and yet retained their pro-apoptotic function in mammalian cells (22, 34). A major question now is which of these two mechanisms for promoting apoptosis is quantitatively more important under physiological conditions where Bax is not artificially overexpressed.

In contrast to Bax, deletion or substitution of the putative channel forming α5 and α6 helices of Bcl-2 abolished its cytoprotective function in both mammalian cells and yeast, indicating that this region is indispensable for function of the Bcl-2 protein. Previously, we reported that deletion of α5 and α6 from Bcl-2 abrogates its ability to form ion channels in liposomes and planar bilayers *in vitro* (12). Thus, it is possible that channel activity is required for Bcl-2 to promote cell survival and diminish Bax-induced cell death. Unfortunately, multiple at-

tempts to produce the Bcl-2-Bax( $\alpha 5\alpha 6$ ) chimeric protein in bacteria for ion channel studies were unsuccessful due to protein instability and insolubility, thus precluding a comparison with the wild-type Bcl-2 protein *in vitro* channel activity.<sup>2</sup> Although we cannot exclude the possibility that substitution the  $\alpha 5\alpha 6$  region of Bcl-2 for the corresponding region of Bax caused a gross misfolding of the protein when expressed in mammalian cells or yeast, the Bcl-2-Bax( $\alpha 5\alpha 6$ ) chimeric protein appeared to be stable, accumulating to levels comparable to the wild-type Bcl-2 protein. Bcl-2-Bax( $\alpha 5\alpha 6$ ) also retained its ability to dimerize with Bax, albeit with reduced efficiency compared with wild-type Bcl-2. Moreover, the Bcl-2-Bax( $\alpha 5\alpha 6$ ) chimera retained the ability to interact with Bcl-2 in yeast two-hybrid assays. Thus, dimerization with Bax or Bcl-2 appears to be insufficient for maintaining the cytoprotective function of the Bcl-2 protein in either mammalian cells or yeast. It will be of interest to identify other proteins with which this Bcl-2-Bax( $\alpha 5\alpha 6$ ) chimera and the wild-type Bcl-2 protein interact. In this regard, Bcl-2 has been reported to bind directly or at least participate in protein complexes containing several types of non-homologous proteins in mammalian cells, including the kinase Raf-1 (35), the phosphatase calcineurin (36), the Hsp70/Hsc70-regulator BAG-1 (37), the caspase-binding protein Bap31 (38), the spinal muscular atrophy protein (SMN) (39), and others (1). Although the CED-4 homolog Apaf-1 (without WD domain) has recently received much attention for its ability to bind both caspases and Bcl-X<sub>L</sub> (40, 41), we have been unable to detect interactions between Bcl-2 and Apaf-1 using numerous experimental approaches,<sup>2</sup> making it unlikely that differential binding of wild-type and chimeric Bcl-2 to Apaf-1 accounts for the ability of the former and failure of the latter to promote cell survival in mammalian cells. Moreover, as the completed genome of *S. cerevisiae* reveals no Apaf-1 homologs or caspases, it is highly unlikely that the cytoprotective function of Bcl-2 observed in yeast is involved in such protein interactions.

Although necessary for function of Bcl-2, the  $\alpha 5\alpha 6$  region appears to be insufficient for promoting cell survival, as replacing the  $\alpha 5\alpha 6$  region of Bax with this segment of Bcl-2 did not convert Bax to a cytoprotective protein. Likewise, although the  $\alpha 5\alpha 6$  region of Bax was necessary for its cytotoxic activity in yeast, engineering these predicted  $\alpha$ -helices into the Bcl-2 protein was insufficient for switching its phenotype. Several previous reports have suggested that the BH3 domain (second  $\alpha$ -helix) is an important determinant of the functions of pro-apoptotic Bcl-2 family proteins in mammalian cells and yeast (1, 24, 27). The BH4 domain (first  $\alpha$ -helix in Bcl-X<sub>L</sub> structure) of anti-apoptotic Bcl-2 family proteins has also been shown to be important for their cytoprotective function in both yeast and mammalian cells (1, 17, 35). Therefore, the opposing phenotypes of Bcl-2 and Bax presumably require both the  $\alpha 5\alpha 6$  region and additional domains such as BH3 or BH4. Determination of the topology of the Bcl-2 and Bax proteins when integrated into membranes in channel-forming conformation will help to reveal whether these other domains such as BH3 and BH4 directly contribute to channel formation by integrating perpendicularly through membranes as proposed for  $\alpha 5$  and  $\alpha 6$ , versus regulating cell death through their contributions to dimerization among Bcl-2 family proteins or interactions

with other types of non-homologous proteins such as those involved in caspase regulation.

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<sup>2</sup> S. Matsuyama, S. L. Schendel, Z. Xie, and J. C. Reed, unpublished observations.